Insulin Signaling and Growth Control in *Drosophila melanogaster*

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Für Flavia und Deborah

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Summary

Despite the fact that increase in size is one of the most obvious features of development, relatively little is known about the mechanisms at work to ensure proper growth at the levels of the cell, the tissues and the whole organism. A genetic approach in the model organism *Drosophila melanogaster* was initiated to shed light on the regulatory network governing growth. To identify genes involved in growth regulation, an unbiased screen for mutations that either stimulate or inhibit growth was performed. The rationale of the screen was to generate mosaic flies consisting of heads largely homozygous for randomly induced mutations on heterozygous bodies. By screening for head size abnormalities in such animals, mutations in genes involved in growth control could be selected for. Flies with heads of reduced size (so-called pinheads) can be expected to carry a lesion in a growth-promoting gene, whereas bigger than normal heads are indicative of loss of a growth-restricting gene function. A detailed complementation analysis with the obtained alleles revealed that the backbone of the signal transduction cascade initiated by the insulin receptor was hit many times. Multiple mutations in the genes encoding the insulin receptor (Inr), the catalytic subunit of the lipid kinase PI3K (Dp110), and the serine-threonine kinase Akt (also called protein kinase B, PKB) were recovered by virtue of the resulting pinhead phenotype, demonstrating the outstanding importance of this signaling pathway in the control of cellular growth. In addition, mutations in a number of novel genes that either promote or dampen growth were identified. Special emphasis was put on the characterization of the gene encoding the *Drosophila* homolog of the small GTPase Rheb (Ras homolog enriched in brain). By means of a detailed epistasis analysis, *Rheb* could be unequivocally placed downstream of the tumor suppressor genes *Tsc1*/*Tsc2* (*Tuberous Sclerosis Complex 1/2*) and upstream of *TOR* (*target of Rapamycin*) and *S6K* (*ribosomal protein S6 kinase*), a finding that was further substantiated by the biochemical demonstration that the activity of S6K critically depends on Rheb.

A second part of this thesis deals specifically with the role of a key component of the insulin receptor signaling pathway, the proto-oncogene Akt (or PKB). Akt is a serine-threonine protein kinase carrying an NH2-terminally located pleckstrin homology (PH) domain that binds with high affinity to D3-phosphorylated phosphatidylinositols (PI(3,4)P2 and PI(3,4,5)P3). Upon signaling activity, the concentration of the second messenger PIP3 in the plasma membrane raises, thereby recruiting Akt to the

membrane where it is activated by two subsequent phosphorylation steps. Using novel protein variants, the phenotypic consequences of impaired Akt function during *Drosophila* development were investigated. Whereas numerous studies of Akt function in mammalian cell culture systems have implicated this kinase as a key player in the protection from apoptosis, loss of *Drosophila* Akt function did not trigger programmed cell death. Cells devoid of Akt function rather grew very slowly, and animals with reduced Akt function emerged with a substantial delay and displayed dramatically reduced body size due to fewer and smaller cells. Most importantly, animals completely lacking the tumor suppressor PTEN were rescued by a mutation in Akt that specifically lowers the affinity of its PH domain for PIP3. Two lessons can be learnt from these rather unexpected survivors. First, activation of Akt is the critical event caused by the loss of PTEN function. Second, the only essential function that PTEN performs is to dephosphorylate PIP3.

Zusammenfassung

Obwohl die Grössenzunahme eine augenfällige Eigenschaft der Entwicklung darstellt, ist über die Mechanismen, welche das Wachstum von Zellen, Geweben und ganzen Organismen steuern, erstaunlich wenig bekannt. Um mehr Licht in die Wachstumskontrolle zu bringen, wurde ein genetischer Ansatz im Modellorganismus *Drosophila melanogaster* gewählt. Eine Mutagenese verfolgte das Ziel, Gene, deren Produkte wachstumsfördernd oder -hemmend wirken, zu identifizieren. Zu diesem Zweck wurden Mosaiktiere generiert, deren Köpfe grösstenteils homozygot für zufällige Mutationen waren, während die Körper heterozygot blieben. Das Auftreten von unnatürlichen Kopfgrössen erlaubte eine Selektion von Mutationen in Genen, die eine Rolle in der Wachstumskontrolle spielen. Von Fliegen mit kleineren Köpfen (sogenannten Pinheads) kann erwartet werden, dass sie eine wachstumsfördernde Genfunktion beeinträchtigt haben, während grössere Köpfe auf einen Defekt in einem wachstumshemmenden Gen hinweisen. Eine detaillierte Komplementationsanalyse mit den gefundenen Allelen hat gezeigt, dass das Rückgrat des Signaltransduktionswegs des Insulinrezeptors mehrfach getroffen worden ist. Mehrere Allele in den Genen, die für den Insulinrezeptor, für die katalytische Untereinheit der Lipidkinase PI3K sowie für die Serin/Threonin-spezifische Proteinkinase Akt (auch Proteinkinase B genannt) kodieren, wurden aufgrund des resultierenden Pinhead-Phänotyps gefunden. Dies belegt eindrücklich die Wichtigkeit dieses Signaltransduktionswegs in der Wachstumskontrolle. Zusätzlich konnten weitere, teilweise unbekannte Gene identifiziert werden, die einen positiven oder negativen Einfluss auf das Wachstum ausüben. Spezielle Aufmerksamkeit wurde der Charakterisierung eines Gens gewidmet, welches für das Fliegenhomologe der kleinen GTPase Rheb kodiert. Mittels detaillierter Epistasie-Analyse konnte *Rheb* zweifelsfrei in einem genetischen Netzwerk unterhalb von den Tumorsuppressorgenen *Tsc1/2* (*Tuberous Sclerosis Complex 1/2*) sowie oberhalb von *TOR* (*Target of Rapamycin*) und *S6K* (*ribosomale Protein S6 Kinase*) eingeordnet werden. Dieser Befund wurde durch biochemische Experimente unterstützt, welche eine Abhängigkeit der S6K-Aktivität von Rheb demonstrieren.
In Mittelpunkt des zweiten Teils der vorliegenden Arbeit steht das Proto-Onkogen Akt. Die Proteinkinase Akt trägt am NH2-terminalen Ende eine Pleckstrin-Homologie (PH) Domäne, welche mit hoher Affinität an Phosphatidylinositole bindet, die an der D3 Position phosphoryliert sind (PI(3,4)P2 und vor allem PI(3,4,5)P3, kurz PIP3). Bei

Aktivierung der Signalkaskade steigt die Konzentration des sekundären Botenstoffes PIP3 in der Zellmembran an, wodurch sich Akt an die Membran anlagert, wo es durch zwei folgende Phosphorylierungsschritte aktiviert wird. Durch den Einsatz von neuen Mutationen im Gen, welches für *Drosophila* Akt kodiert, konnten die phänotypischen Auswirkungen einer verminderten Akt Aktivität während der Entwicklung untersucht werden. Die Ergebnisse von zahlreichen Studien an Zellkulturen von Säugern haben auf eine wichtige anti-apoptotische Funktion von Akt hingewiesen. Deshalb mag es erstaunen, dass der Verlust der Akt Funktion während der Entwicklung von *Drosophila* keinen programmierten Zelltod auslöst. Vielmehr wachsen die betroffenen Zellen äusserst langsam, und Tiere mit beeinträchtigter Akt Funktion erreichen das Adultstadium mit beträchtlicher Verspätung und weisen eine deutlich reduzierte Körpergrösse auf, die aufgrund von weniger und kleinerern Zellen zustande kommt. Interessanterweise können Fliegen ohne die Funktion des Tumorsuppressorgens PTEN überleben, wenn sie in Akt eine Mutation tragen, welche die Affinität der PH Domäne für PIP3 vermindert. Diese überlebenden Tiere demonstrieren zwei wichtige Befunde: Die Aktivierung der Proteinkinase Akt scheint das einzige kritische Ereignis zu sein, das durch den Funktionsverlust von PTEN bewirkt wird. Und zweitens kann gefolgert werden, dass PTEN keine weiteren Funktionen als die Dephosphorylierung von PIP3 ausübt.

Introduction

No life without growth. Every living being needs to grow, at least during a certain period of its existence. Even the simplest single-celled organisms require growth for their duplication. They have to attain a certain size before dividing and thereby giving rise to two daughter cells. In multicellular organisms, the growth of a multitude of cells must be highly orchestrated to guarantee the proper development of tissues, organs, and complex bodies. Growth can be seen as an accumulation of biomass. It is reflected in the multiplication of cells of a certain size. The number of cells is determined by the relative rates of proliferation and cell death (Conlon and Raff, 1999). Traditionally, research in the growth control field focussed mainly on the regulation of the cell division cycle. Considerable advances in our understanding of the cellular cell cycle machinery have resulted from these efforts. In the 1990s, the role of programmed cell death as a means of constraining tissue size got appreciated. A plethora of recent studies has contributed to unraveling the tangled web of cellular suicide pathways. The regulation of a cell's biosynthesis capacity, the basis for cellular growth, has received little attention until recently. Two elegant studies in Drosophila resulted in the revival of the long-known concept of predominance of growth over cell cycle progression (Neufeld et al., 1998; Weigmann et al., 1997). By manipulating the cell cycle length during larval wing development, it could be convincingly demonstrated that neither acceleration nor retardation of the cell cycle progression did influence net growth. These findings undoubtedly caused a shift in the central issues of growth control. A main challenge will be to uncover the signaling pathways that control the rate of biosynthesis in accordance with the environmental conditions. The goal of the present thesis was to take a genetic approach in elucidating this regulatory framework in the model organism *Drosophila melanogaster*, with a special emphasis on the role of signaling downstream of the insulin receptor.

Signal transduction pathways and growth control

An overview on some signaling pathways that contribute to the regulation of growth is depicted in:

Genetic control of cell size

Hugo Stocker and Ernst Hafen

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Genetic control of cell size

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Over the past 25 years, the genetic control of cell size has mainly been addressed in yeast, a single-celled organism. Recent insights from *Drosophila* have shed light on the signalling pathways responsible for adjusting and maintaining cell size in metazoans. Evidence is emerging for a signalling cascade conserved in evolution that links external nutrient sources to cell size.

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Abbreviations

dMyc	*Drosophila* Myc
dS6K	*Drosophila* p70 S6 kinase
IGF	insulin-like growth factor
Inr	insulin receptor
IRS	insulin receptor substrate
MAPK	mitogen-activated protein kinase
PIP3	phosphatidylinositol 3,4,5 trisphosphate
PI3K	phosphoinositide 3-kinase
PKB	protein kinase B
PTEN	phosphatase and tensin homologue
RTK	receptor tyrosine kinase
TOP	terminal oligopyrimidine tract
TOR	target of rapamycin
uORF	upstream open reading frame

Introduction

During metazoan development, cells undergo dramatic changes in size and shape, yet each of the diverse cell types attains a characteristic size upon differentiation, indicating that cell size must be under tight genetic control. Some determinants of cell size have been known for decades. First, the size of a cell critically depends on its DNA content. A correlation between the number of chromosome sets and cell size has been demonstrated in organisms as diverse as yeast, *Drosophila*, salamanders and mice [1–5]. Furthermore, during development, many animal and plant species generate large specialised cell types that are polyploid as a consequence of several rounds of endoreplication. In nematodes, polyploidization of hypodermal cells contributes significantly to body-size control [6]. Second, cell size is strongly influenced by the environment. Under adverse nutritional conditions, for example, yeast cells divide at a smaller size than they normally do [1]. This correlation also holds true for multicellular organisms such as *Drosophila* [7,8]. Another determinant of cell size is temperature: laboratory fruit flies are bigger when reared at low temperature owing to an increase in cell size [9–12].

Despite the long-standing interest in the phenomenon of cell-size control, the underlying genetic networks are still poorly understood. Elegant experiments in yeast pioneered genetic analysis in this area and revealed the predominance of cellular growth (equating to an increase in mass) over the cell-cycle machinery [13–15]. Whereas blocking cell-cycle progression does not impede cellular growth, interfering with general biosynthesis results in cell cycle arrest. Conversely, mutations that accelerate the cell cycle do not promote growth but rather result in progressively smaller cells. The issues of how cellular growth is regulated and how a cell senses the critical size at which it should divide have remained largely unresolved.

The aim of this review is to highlight recent advances from genetically amenable model organisms in understanding the signalling pathways governing cellular growth and the mechanisms that co-ordinate growth with cell division.

Signal transduction pathways controlling growth

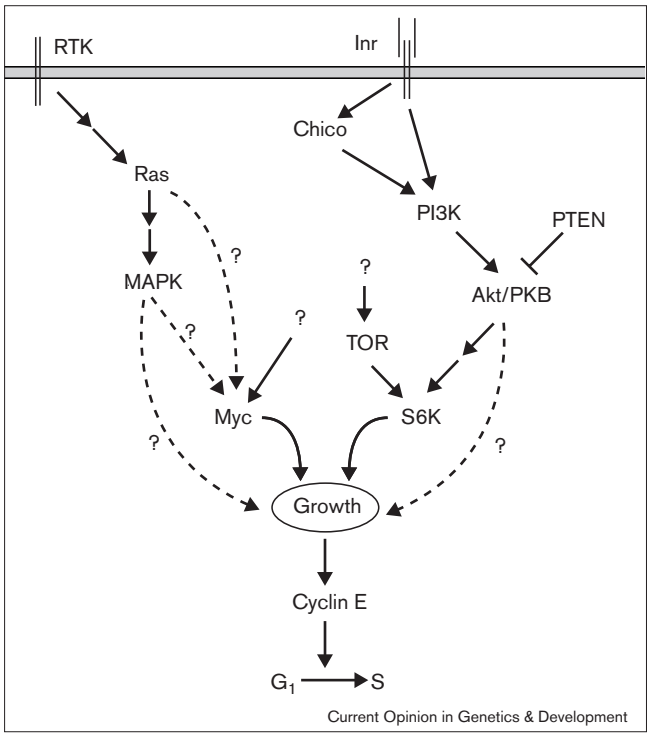
The past year has seen an unprecedented wealth of studies on growth regulation in the fruit fly *Drosophila melanogaster*. Figure 1 depicts the various signalling pathways that are currently attracting considerable attention.

Insulin receptor signalling in *Drosophila*

Flies lacking the function of Chico, a homologue of the human insulin receptor substrates 1–4 (IRS1–4), are small as a result of a reduction in both cell number and size [16••]. Cells mutant for *chico* grow more slowly than heterozygous cells and are smaller throughout development. The analysis of mutations in other components of the insulin receptor (Inr) signal transduction cascade has revealed strikingly similar phenotypes. For example, heteroallelic combinations of *Inr* alleles result in small flies with fewer and smaller cells [16••,17,18]. Furthermore, cells devoid of the lipid kinase PI3K show a pronounced impairment of growth and a reduction in size whereas overexpression of a wild-type or of an activated version of the catalytic subunit of PI3K stimulates cellular growth, resulting in bigger cells [19,20••]. PTEN is a lipid phosphatase that antagonises the activity of PI3K. Loss of *Drosophila* PTEN (dPTEN) function results in enhanced growth and larger cells [21••–23••]. Conversely, overexpression of dPTEN reduces cell number and cell size [21••–23••].

The balance of PI3K and PTEN activities determines the level of the second messenger PIP3. What might be the effectors of PIP3 in *Drosophila* growth control? Overexpression studies suggest that the serine/threonine kinase Dakt/PKB plays a critical role downstream of PI3K. As has been shown for PI3K, overexpression of Dakt/PKB in clones increases cell size without affecting cell number [24•]. Accordingly, loss of Dakt/PKB function results in impaired growth and small cells ([24•]; H Stocker,

Figure 1



Schematic representation of signalling pathways controlling cellular growth (protein synthesis) in *Drosophila*. This network contributes to the control of protein synthesis. The transcriptional regulation of dMyc as well as the control of dTOR activity remain enigmatic. Cellular growth might be coupled to the cell-cycle machinery via Cyclin E; however, Myc probably also acts directly on cell-cycle components.

E Hafen, unpublished data). Flies homozygous for hypomorphic *Dakt/PKB* alleles resemble *chico* mutant flies (H Stocker, E Hafen unpublished data). Furthermore, cells lacking dPTEN function grow to a normal size in a background with reduced Dakt/PKB activity [23••].

Phosphorylation of the ribosomal protein S6 by S6 kinase (S6K) leads to an increase in the translation of 5′ TOP (terminal oligopyrimidine tract) mRNAs that largely encode components of the translational apparatus such as ribosomal proteins [25]. As S6K activity can be regulated by PI3K in mammals (reviewed in [26]), mutations in *dS6K* might be expected to yield similar phenotypes as mutations in other components of the Inr/PI3K pathway. Indeed, flies lacking dS6K function are viable, develop slowly and are small [27••]. Remarkably, the size reduction is caused exclusively by a decrease in cell size [27••].

Nutrition and growth control

Starvation of larvae phenocopies the small-size phenotype associated with mutations in the Inr signalling pathway [7,8], suggesting that this signalling relays information on the availability of nutrients to every single cell. But what are the ligands that control the activity of the insulin receptor and how is their expression regulated? The essentially complete *Drosophila* genome sequence [28] has revealed the presence of at least seven insulin-like peptides. Interestingly, overexpression of one such peptide produces bigger flies with more and bigger cells (W Brogiolo, E Hafen, unpublished data).

It is worth noting that a highly conserved Inr pathway also operates in the nematode *Caenorhabditis elegans* to control lifespan and an alternative larval stage, called the dauer larva, in response to suboptimal food conditions [29–31]. However, there are two major differences in how the Inr-signalling pathway is used in the fly and the nematode. Whereas Inr signalling acts in a cell-autonomous fashion in *Drosophila*, it is required only in a subset of neurons in *C. elegans* [32]. Furthermore, no effects of Inr signalling on cell size have been reported in the nematode. Nevertheless, it is tempting to speculate that the evolutionarily ancient function of Inr signalling is to coordinate development with nutritional conditions. The discrepancies between *C. elegans* and *Drosophila* could be a consequence of the underlying developmental mechanisms. In contrast to the fly, the organ and body size of the nematode do not rely on growth and proliferation of precursor tissues, but rather emerge as a result of a strict developmental programme (cell lineage).

The regulation of protein synthesis in response to the availability of nutrients is likely to represent a fundamental mechanism of growth control in all eukaryotes. In yeast, the TOR proteins play a key role in this process. Upon nutrient deprivation, yeast cells downregulate TOR function which results in a dampening of translational activity as well as an induction of autophagy [33]. The Inr signalling pathway may contribute to the control of TOR activity in multicellular eukaryotes. In fact, in *Drosophila*. cells devoid of TOR function grow poorly (S Oldham, E Hafen, unpublished data).

Inr signalling in vertebrates

The paramount importance of insulin and the insulin-like growth factors (IGFs) in mammalian growth control is well documented (reviewed in [34]). Mice lacking any one of either IGF-I, IGF-II, IGF1 receptor, Inr, IRS1 or IRS2 have a reduced body size. Moreover, the cell cycle is dramatically prolonged in embryonic fibroblasts derived from *Igf1 receptor* knockout mice [35], as in flies with reduced Inr signalling activity ([27••]; H Stocker, E Hafen, unpublished observations). However, only recently have studies in mice investigated the effects of IGF signalling on cell size. Such an analysis of *igf-I* knockout mice has revealed a reduced muscle fibre size in the diaphragm [36]. Consistently, treatment of myotubes with IGF-I results in hypertrophy, and this effect can be mimicked by expression of activated Akt/PKB [37]. Furthermore, modulating the activity of PI3K during heart development affects both cell and organ size [38•].

Myc functions in growth control

Whereas numerous functions in mammalian cell proliferation control have already been ascribed to the

proto-oncogene c-*myc* [39], recent experiments in *Drosophila* suggest additional direct roles in growth control. The mutation *diminutive*, which results in a reduced body size and slender bristles, is caused by a defect in *Drosophila* Myc (dMyc) [40,41]. The study of additional mutations revealed that whereas only cell size is reduced in adult flies homozygous for one hypomorphic allele, the cell-size reduction is accompanied by a decrease in cell number in a more severe mutation [42••]. dMyc-overexpressing cells are substantially larger than wild-type ones, independent of the cell-cycle phase [42••]. The suggested growth-promoting capacity of Myc recently received strong support by two studies demonstrating a similar role in B cells [43•,44•].

How might Myc exert its growth-enhancing function? Among the proposed Myc targets, there are a number of genes involved in translational control and metabolism [45–48]. Notably, the mRNA cap-binding protein eIF4E — a key regulator of translational initiation [49] — contains two Myc-binding sites in its promoter [50]. Furthermore, the only *bona fide* Myc target gene in *Drosophila*, *pitchoune*, encodes a DEAD box RNA helicase that may be involved in ribosome biogenesis [51].

Ras-MAPK signalling

In model organisms, Ras-MAPK signalling has been predominantly associated with promoting changes in cell fate during development. Cells that are homozygous mutant for partial loss-of-function mutations in various components of the Ras-MAPK signal transduction cascade, however, also grow poorly and stay small [52]. This observation has now been complemented by a study on the growth-enhancing function of Ras [53••]. As for dMyc, expression of activated Ras augments cellular growth and increases cell size. Interestingly, Ras appears to upregulate dMyc at a post-transcriptional level, consistent with the previous finding that Ras enhances Myc protein stability in vertebrate tissue-culture cells [54]. A growth-promoting activity of Ras has also been described in mice, where Ras hyperactivity causes cardiac hypertrophy (reviewed in [55]).

Gigas – a cell size mutation affecting ploidy

The *Drosophila* gene *gigas* encodes a homologue of the human tumour suppressor gene *TSC2* (tuberous sclerosis complex gene 2), a putative exchange factor for the small GTPase Rap1. Cells that are mutant for *gigas* are very large, possibly as a consequence of endoreplication [56]. Despite their increased size, they are capable of differentiating properly, as are the giant cells in human hamartomas caused by the lack of one copy of *TSC2* [56].

NF1 – a component of the humoral control?

All the above-mentioned growth regulators act in a strictly cell-autonomous manner. In contrast, in mutants for the *Drosophila* homologue of the tumour suppressor NF1 (neurofibromatosis type 1), the cell-size reduction is non-autonomous. Whereas the wing cells are smaller in homozygous mutant flies, mutant cells are of normal size when surrounded by heterozygous cells [57]. Expression of activated Protein Kinase A suffices to restore wild-type cell size in homozygous mutant flies [57]. As NF1 is also required for the proper response to certain neuropeptides and for learning [58,59], it is conceivable that NF1 acts in the nervous system to regulate expression of unknown hormone(s) which, in turn, coordinate growth.

Coupling of cell growth to cell-cycle progression

Insights from yeast

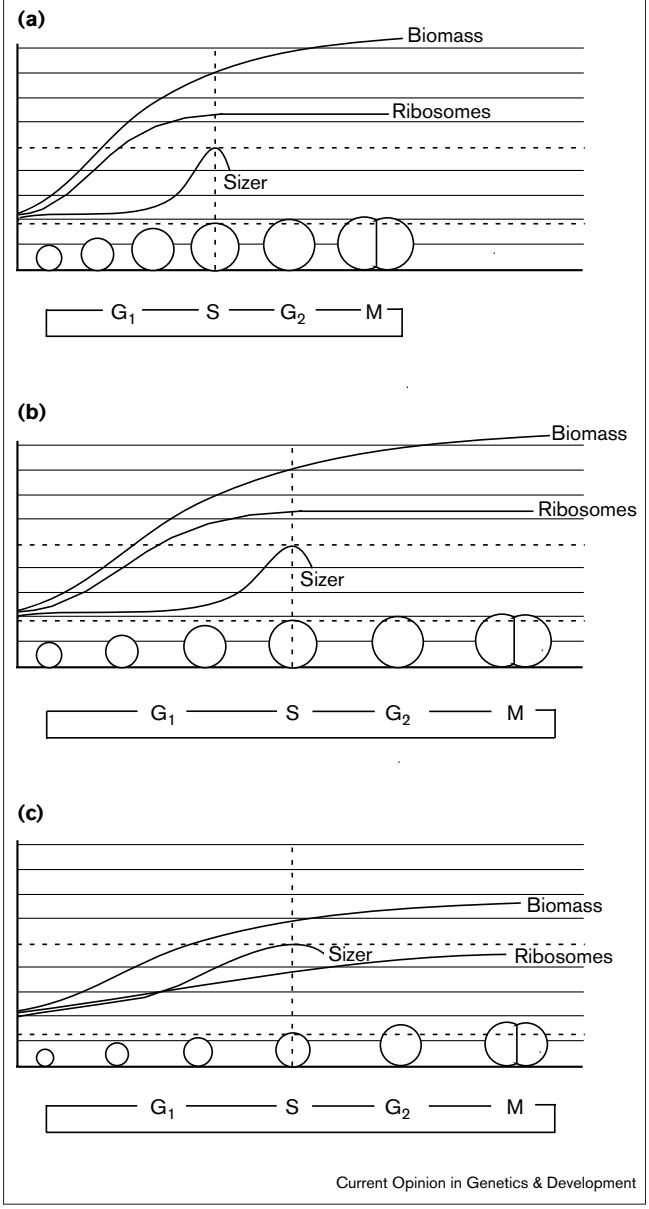
In the *Drosophila* mutants discussed above, reductions in cell size are paralleled by a decreased growth rate (with the possible exception of *NF1*). This raises the issue of how cell proliferation is linked to growth — that is, how does a cell sense the critical size at which it has to initiate cell division? Genetic analyses in yeast have shed light on some basic mechanisms. In the budding yeast *Saccharomyces cerevisiae*, a growth-sensing mechanism operates during late G_1 phase (START). Translational control of the G_1 cyclin Cln3 is achieved by means of an upstream ORF (uORF) in the 5′ leader sequence which ensures that Cln3 fails to accumulate under poor growth conditions. As Cln3 acts at the top of a hierarchy of genes controlling the START transcriptional programme, translational regulation of this very unstable protein could serve as a growth sensor [60,61]. Furthermore, the influence of ploidy on cell size has been investigated at the level of transcription in *S. cerevisiae* [62•]. A microarray-based analysis of gene expression in isogenic strains that varied only in ploidy showed a relative repression of the G_1 cyclins Cln1 and Pcl1 in cells with high ploidy. As expression levels of G_1 cyclins determine the passage through START, ploidy-dependent repression of G_1 cyclins may explain why cells with more chromosome sets pass through START at a larger size.

In contrast, mitotic control links growth to the cell cycle in wild-type cells of the fission yeast *Schizosaccharomyces pombe*. The rate-limiting component for G_2–M progression, the phosphatase Cdc25, appears to be translationally controlled in a manner very analogous to Cln3 in *S. cerevisiae* ([63•] but see also [64,65]). Under normal conditions, G_1–S size control is cryptic in *S. pombe* because cells are already larger than the threshold size after completion of mitosis. The analysis of *cig1*, *cig2* and *puc1* triple mutant cells, however, revealed the importance of these G_1 cyclins in G_1–S size control [66].

Co-ordination of growth and cell cycle in *Drosophila*

Are similar control mechanisms to those in yeast at work in multicellular organisms? Again, recent experiments in *Drosophila* provide some answers. Cyclin E and String, a Cdc25 homologue, are rate-limiting components in G_1–S and G_2–M progression, respectively [67,68]. Overexpression of Cyclin E shortens G_1 phase but leads to a concomitant increase in G_2 phase length. Conversely, overexpression of String reduces G_2 while prolonging G_1 [69]. Therefore G_1–S and G_2–M progression appear to be controlled in a different manner, and reducing the length of one gap phase of the cell cycle results in a compensatory extension of the other. To

Figure 2



Model for growth-dependent G_1–S control. **(a)** If the availability of nutrients is not limiting, cells exiting mitosis first focus on replenishing the ribosome pools. High levels of Inr and S6K activity lead to selective translation of 5′ TOP mRNAs required for ribosome biogenesis. The sizer protein (possibly Cyclin E in *Drosophila*) accumulates poorly in this initial phase but rises rapidly as soon as the full translational capacity is achieved. **(b)** Mutations in genes encoding ribosomal proteins (*Minutes*) impair the general translational efficiency. Hence, the rate of increase in biomass is reduced and cell-cycle length is prolonged. The completion of ribosome biogenesis, however, is still a prerequisite for the accumulation of the sizer protein: thus, cells progress through the cell cycle at the same critical size. **(c)** As Inr and S6K activities are low under adverse nutritional conditions, ribosomal protein mRNAs are no longer preferentially translated: thus, accumulation of the sizer protein commences earlier and reaches the critical concentration at a smaller cell size. The same scenario applies to mutations reducing Inr signalling activity. In addition, a similar mechanism might control G_2–M progression.

accelerate the cell cycle, both gap phases need to be shortened, for example by co-expression of Cyclin E and String [69]. A similar effect can be achieved by overproduction of the transcriptional regulator E2F. In both cases, cell size decreases as cell-division rate rises, leaving total growth (clonal size) constant [69]. These experiments elegantly demonstrated the inability of cell-cycle acceleration to stimulate growth, as is the case in yeast.

Studying the cell-cycle profiles of the various growth mutants has enabled a further dissection of the link between cellular growth and cell-cycle progression. Cells with reduced Inr signalling activity (e.g. overexpressing dPTEN or mutant for *dS6K*) show a considerable increase in cell-cycle length, yet their cell cycle profiles look surprisingly normal [23••,27••]. Hence, all the phases of the cell cycle are proportionally extended. Conversely, augmenting cellular growth (e.g. by overexpression of PI3K, dMyc or activated Ras) shortens G_1 phase but is insufficient to override G_2-M control [20••,42••,53••]. As for overexpression of Cyclin E, cell-cycle acceleration requires co-expression of String. Since the *string* gene bears extensive *cis*-regulatory sequences that respond to various developmental cues [70,71], *string* is an attractive candidate to couple patterning signals to cell-cycle progression [42••,53••].

How does the G_1–S control respond to cellular growth? By analogy to *S. cerevisiae*, one might expect a G_1 cyclin to be tightly controlled at the translational level. Indeed, Cyclin E fulfils the criteria for a growth sensor. First, Cyclin E levels are rate-limiting for G_1–S progression [67,69]. Second, the 5′ region of the *cyclin E* mRNA contains several uORFs, comparable to *cln3* [72]. As Cyclin E is unstable, accumulation to a threshold level requires high translational activity. Third, increasing growth rate by overexpression of either Myc or activated Ras results in an upregulation of Cyclin E protein [53••].

Reducing translational efficiency, however, does not always equate with cell-size reduction in *Drosophila*. Mutations in ribosomal proteins — known as *Minutes*, reviewed in [73] — that impair ribosome biogenesis and slow down development do not appreciably reduce cell size [27••,69,73]. The role of S6K may be key in understanding this apparent inconsistency. By selectively promoting the translation of 5′ TOP mRNAs encoding ribosomal proteins and other components of the translational apparatus, S6K activity contributes to prevent the accumulation of cell-cycle regulators until the cell has reached an appropriate size [74]. Whereas translation is globally decreased in *Minute* mutants, non-ribosomal protein mRNAs would be preferentially translated in cells devoid of dS6K. This could allow a cell-cycle regulator to accumulate relatively rapidly, resulting in cell division at a smaller size [74]. A schematic representation of this model is shown in Figure 2.

The emerging picture of a growth-sensing G_1–S control and a patterning-dependent G_2–M control is attractive but

incomplete. Although it can explain the phenotypes associated with growth stimulation (e.g. by overexpressing dMyc or activated Ras), it cannot account for the proportional extension of all cell-cycle phases in cells with impaired growth rates (e.g. mutant for *dS6K* or overexpressing dPTEN, respectively). Therefore, we propose the existence of a growth-dependent G_2–M control that remains cryptic during normal development but becomes apparent once the translational capacity of a cell drops below a certain threshold. This two-step mechanism would be reminiscent of size control in fission yeast where the mitotic control is composed of a 'sizer' and a 'timer' [64].

Concluding remarks

Recent experiments in genetically tractable model organisms are helping to unravel the mechanisms that control cellular growth and how it is co-ordinated with cell-cycle progression. In particular, a well-conserved signalling pathway leading from the insulin receptor to the translational machinery has been identified on the basis of *Drosophila* growth phenotypes. This pathway has been subject to extensive biochemical characterisation in mammalian cells but it remains unclear whether genetic manipulation of Inr signalling in mammals results in cell-size phenotypes comparable to those observed in *Drosophila*. The cell-size issue has possibly received too little attention to date, and it may also be more difficult to study cell size in mammalian tissues. Alternatively, cell size could be more rigorously controlled in mammals. The large differences in body size observed in mammals (such as mice and elephants) are mainly the result of varying cell number [75], whereas cell size contributes significantly to body-size variations between *Drosophila* species [76]. Cellular growth and cell division rate may be more tightly coupled in mammals than in flies, rendering the distinction between growth control and cell-cycle control more difficult to analyse. *Drosophila* may, therefore, be a particularly well-suited model system for establishing the basic mechanisms of cell size and growth control.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Nurse P: **The genetic control of cell volume.** In *The Evolution of Genome Size*. Edited by Cavalier-Smith T. New York: John Wiley and Sons; 1985:185-196.

2. Dobzhansky T: **The influence of the quantity and the quality of the chromosomal material on the size of cells in *Drosophila melanogaster*.** *Arch Entwicklungsmech Organ* 1929, **115**:363-379.

3. Santamaria P: **Analysis of haploid mosaics in *Drosophila*.** *Dev Biol* 1983, **96**:285-295.

4. Fankhauser G: **Maintenance of normal structure in heteroploid salamander larvae, through compensation of changes in cell size by adjustment in cell number and cell shape.** *J Exptl Zool* 1945, **100**:445-455.

5. Henery CC, Bard JB, Kaufman MH: **Tetraploidy in mice, embryonic cell number, and the grain of the developmental map.** *Dev Biol* 1992, **152**:233-241.

6. Flemming AJ, Shen ZZ, Cunha A, Emmons SW, Leroi AM: **Somatic polyploidization and cellular proliferation drive body size evolution in nematodes.** *Proc Natl Acad Sci USA* 2000, **97**:5285-5290.

7. Robertson FW: **The ecological genetics of growth in *Drosophila*. VI. The genetic correlation between the duration of the larval period and body size in relation to larval diet.** *Genet Res* 1963, **4**:74-92.

8. Simpson P: **Parameters of cell competition in the compartments of the wing disc of *Drosophila*.** *Dev Biol* 1979, **69**:182-193.

9. Alpatov WW: **Phenotypical variation in body and cell size of *Drosophila melanogaster*.** *Biological Bulletin* 1930, **58**:85-103.

10. Robertson FW: **Studies in quantitative inheritance. XII. Cell size and number in relation to genetic and environmental variation of body size in *Drosophila*.** *Genetics* 1959, **44**:869-896.

11. Delcour J, Lints FA: **Environmental and genetic variations of wing size, cell size, and cell division rate in *Drosophila melanogaster*.** *Genetica* 1966, **37**:543-556.

12. Partridge L, Barrie B, Fowler K, French V: **Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature.** *Evolution* 1994, **48**:1269-1276.

13. Hartwell LH: **Genetic control of the cell division cycle in yeast: II. genes controlling DNA replication and its initiation.** *J Mol Biol* 1971, **59**:183-194.

14. Nurse P: **Genetic control of cell size at cell division in yeast.** *Nature* 1975, **256**:547-551.

15. Johnston GC: **Cell size and budding during starvation of the yeast *Saccharomyces cerevisiae*.** *J Bacteriol* 1977, **132**:738-739.

16. •• Bohni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andruss BF, Beckingham K, Hafen E: **Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4.** *Cell* 1999, **97**:865-875.
This study provides a genetic analysis of the IRS1–4 homologue in *Drosophila*. A role for the insulin receptor signalling pathway in the autonomous control of growth and cell size is described.

17. Fernandez R, Tabarini D, Azpiazu N, Frasch M, Schlessinger J: **The *Drosophila* insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential.** *EMBO J* 1995, **14**:3373-3384.

18. Chen C, Jack J, Garofalo RS: **The *Drosophila* insulin receptor is required for normal growth.** *Endocrinology* 1996, **137**:846-856.

19. Leevers SJ, Weinkove D, MacDougall LK, Hafen E, Waterfield MD: **The *Drosophila* phosphoinositide 3-kinase Dp110 promotes cell growth.** *EMBO J* 1996, **15**:6584-6594.

20. •• Weinkove D, Neufeld TP, Twardzik T, Waterfield MD, Leevers SJ: **Regulation of imaginal disc cell size, cell number and organ size by *Drosophila* class I(A) phosphoinositide 3-kinase and its adaptor.** *Curr Biol* 1999, **9**:1019-1029.
The authors report on the generation of null alleles in the regulatory (p60) and the catalytic (Dp110) subunit of the class(I)A PI3K, respectively. The loss-of-function phenotypes demonstrate that both genes are essential and that they are autonomously required in imaginal disc cells to attain wild-type size. Furthermore, manipulating the activity of Dp110 affects cell size throughout the cell cycle.

21. •• Goberdhan DCI, Paricio N, Goodman EC, Mlodzik M, Wilson C: ***Drosophila* tumor suppressor *PTEN* controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway.** *Genes Dev* 1999, **13**:3244-3258.
See annotation [23••].

22. •• Huang H, Potter CJ, Tao W, Li DM, Brogiolo W, Hafen E, Sun H, Xu T: **PTEN affects cell size, cell proliferation and apoptosis during *Drosophila* eye development.** *Development* 1999, **126**:5365-5372.
See annotation [23••].

23. •• Gao X, Neufeld TP, Pan D: ***Drosophila* PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways.** *Dev Biol* 2000, **221**:404-418.
The experiments described in these references [21••–23••] demonstrate that the tumour suppressor dPTEN, a lipid phosphatase, antagonises Inr signalling to control growth and cell size during *Drosophila* development.

24. Verdu J, Buratovich MA, Wilder EL, Birnbaum MJ: **Cell-autonomous**
• **regulation of cell and organ growth in *Drosophila* by Akt/PKB.** *Nat Cell Biol* 1999, **1**:500-506.
The authors investigate the role of Akt/PKB in *Drosophila* growth control. On the basis of overexpression phenotypes, they propose that Akt/PKB acts downstream of PI3K and that there exists a bifurcation point immediately upstream of Akt/PKB in the Inr/PI3K signalling cascade.

25. Jefferies HB, Reinhard C, Kozma SC, Thomas G: **Rapamycin selectively represses translation of the 'polypyrimidine tract' mRNA family.** *Proc Natl Acad Sci USA* 1994, **91**:4441-4445.

26. Thomas G, Hall MN: **TOR signalling and control of cell growth.** *Curr Opin Cell Biol* 1997, **9**:782-787.

27. Montagne J, Stewart MJ, Stocker H, Hafen E, Kozma SC, Thomas G:
•• ***Drosophila* S6 kinase: a regulator of cell size.** *Science* 1999, **285**:2126-2129.
This study is the first report of a mutation that causes a body size reduction by exclusively affecting cell size. Cells devoid of S6K function grow poorly and divide at a smaller size than normal. Homozygous mutant animals are severely delayed in development but reach a wild-type number of cells.

28. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF *et al.*: **The genome sequence of *Drosophila melanogaster*.** *Science* 2000, **287**:2185-2195.

29. Cassada RC, Russell RL: **The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*.** *Dev Biol* 1975, **46**:326-342.

30. Riddle DL: **The Dauer Larva.** In *The Nematode* Caenorhabditis elegans. Edited by Wood WN. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1988:393-412.

31. Gottlieb S, Ruvkun G: **daf-2, daf-16 and daf-23: genetically interacting genes controlling Dauer formation in *Caenorhabditis elegans*.** *Genetics* 1994, **137**:107-120.

32. Apfeld J, Kenyon C: **Cell nonautonomy of *C. elegans* daf-2 function in the regulation of diapause and life span.** *Cell* 1998, **95**:199-210.

33. Dennis PB, Fumagalli S, Thomas G: **Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation.** *Curr Opin Genet Dev* 1999, **9**:49-54.

34. Efstratiadis A: **Genetics of mouse growth.** *Int J Dev Biol* 1998, **42**:955-976.

35. Sell C, Dumenil G, Deveaud C, Miura M, Coppola D, DeAngelis T, Rubin R, Efstratiadis A, Baserga R: **Effect of a null mutation of the insulin-like growth factor I receptor gene on growth and transformation of mouse embryo fibroblasts.** *Mol Cell Biol* 1994, **14**:3604-3612.

36. Fournier M, Lewis MI: **Influences of IGF-I gene disruption on the cellular profile of the diaphragm.** *Am J Physiol Endocrinol Metab* 2000, **278**:E707-E715.

37. Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, Moelling K, Yancopoulos GD, Glass DJ: **Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt.** *Science* 1999, **286**:1738-1741.

38. Shioi T, Kang PM, Douglas PS, Hampe J, Yballe CM, Lawitts J,
• Cantley LC, Izumo S: **The conserved phosphoinositide 3-kinase pathway determines heart size in mice.** *EMBO J* 2000, **19**:2537-2548.
This paper addresses the role of PI3K activity in mammalian organ growth control. Transgenic mice expressing constitutively active or dominant-negative PI3K, specifically in the heart, develop either larger or smaller hearts, respectively. The size defects can be explained by differences in myocyte size. These results suggest that the growth-promoting function of PI3K is evolutionarily conserved.

39. Amati B, Alevizopoulos K, Vlach J: **Myc and the cell cycle.** *Front Biosci* 1998, **3**:D250-D268.

40. Gallant P, Shiio Y, Cheng PF, Parkhurst SM, Eisenman RN: **Myc and Max homologs in *Drosophila*.** *Science* 1996, **274**:1523-1527.

41. Schreiber-Agus N, Stein D, Chen K, Goltz JS, Stevens L, DePinho RA: ***Drosophila* Myc is oncogenic in mammalian cells and plays a role in the diminutive phenotype.** *Proc Natl Acad Sci USA* 1997, **94**:1235-1240.

42. Johnston LA, Prober DA, Edgar BA, Eisenman RN, Gallant P:
•• ***Drosophila myc* regulates cellular growth during development.** *Cell* 1999, **98**:779-790.
This is the first demonstration that Myc's primary function is to control cellular growth. Reduction of Myc function during *Drosophila* development reduces growth and cell size. Consistently, overexpression of dMyc promotes growth and increases cell size. Although dMyc drives cells through G_1–S, it is not sufficient to promote G_2–M progression. Furthermore, the authors demonstrate that the signalling molecule wingless is able to repress *dMyc* expression, suggesting a possible link between patterning and growth control.

43. Iritani BM, Eisenman RN: **c-Myc enhances protein synthesis and**
• **cell size during B lymphocyte development.** *Proc Natl Acad Sci USA* 1999, **96**:13180-13185.
See annotation [44•].

44. Schuhmacher M, Staege MS, Pajic A, Polack A, Weidle UH,
• Bornkamm GW, Eick D, Kohlhuber F: **Control of cell growth by c-Myc in the absence of cell division.** *Curr Biol* 1999, **9**:1255-1258.
These reports [43•,44•] underscore the importance of Myc in growth control. Overexpression of Myc increases B cell size during all phases of the cell cycle and is accompanied by an increase in protein synthesis.

45. Dang CV: **c-Myc target genes involved in cell growth, apoptosis, and metabolism.** *Mol Cell Biol* 1999, **19**:1-11.

46. Schmidt EV: **The role of c-myc in cellular growth control.** *Oncogene* 1999, **18**:2988-2996.

47. Greasley PJ, Bonnard C, Amati B: **Myc induces the nucleolin and BN51 genes: possible implications in ribosome biogenesis.** *Nucleic Acids Res* 2000, **28**:446-453.

48. Coller HA, Grandori C, Tamayo P, Colbert T, Lander ES, Eisenman RN, Golub TR: **Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion.** *Proc Natl Acad Sci USA* 2000, **97**:3260-3265.

49. Sonenberg N, Gingras AC: **The mRNA 5′ cap-binding protein eIF4E and control of cell growth.** *Curr Opin Cell Biol* 1998, **10**:268-275.

50. Jones RM, Branda J, Johnston KA, Polymenis M, Gadd M, Rustgi A, Callanan L, Schmidt EV: **An essential E box in the promoter of the gene encoding the mRNA cap-binding protein (eukaryotic initiation factor 4E) is a target for activation by c-myc.** *Mol Cell Biol* 1996, **16**:4754-4764.

51. Zaffran S, Chartier A, Gallant P, Astier M, Arquier N, Doherty D, Gratecos D, Semeriva M: **A *Drosophila* RNA helicase gene, *pitchoune*, is required for cell growth and proliferation and is a potential target of d-Myc.** *Development* 1998, **125**:3571-3584.

52. Diaz-Benjumea FJ, Hafen E: **The sevenless signalling cassette mediates *Drosophila* EGF receptor function during epidermal development.** *Development* 1994, **120**:569-578.

53. Prober DA, Edgar BA: **Ras1 promotes cellular growth in the**
•• ***Drosophila* wing.** *Cell* 2000, **100**:435-446.
Here, the role of the small GTPase Ras1 in growth control in the *Drosophila* wing is described. Cells devoid of Ras1 function are smaller, grow poorly and are out-competed by wild-type cells. Overexpression of activated Ras1 causes an increase in cell size and growth rate. Although activated Ras1 promotes G_1–S progression, cell-cycle length remains constant because of a compensatory lengthening of the G_2 phase. Further experiments suggest that Ras1 exerts these effects at least in part by elevating dMyc protein levels post-transcriptionally. Ras1 and dMyc are also shown to upregulate Cyclin E protein level.

54. Sears R, Leone G, DeGregori J, Nevins JR: **Ras enhances Myc protein stability.** *Mol Cell* 1999, **3**:169-179.

55. Hefti MA, Harder BA, Eppenberger HM, Schaub MC: **Signaling pathways in cardiac myocyte hypertrophy.** *J Mol Cell Cardiol* 1997, **29**:2873-2892.

56. Ito N, Rubin GM: ***gigas*, a *Drosophila* homolog of tuberous sclerosis gene product-2, regulates the cell cycle.** *Cell* 1999, **96**:529-539.

57. The I, Hannigan GE, Cowley GS, Reginald S, Zhong Y, Gusella JF, Hariharan IK, Bernards A: **Rescue of a *Drosophila NF1* mutant phenotype by protein kinase A.** *Science* 1997, **276**:791-794.

58. Guo HF, The I, Hannan F, Bernards A, Zhong Y: **Requirement of *Drosophila* NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides.** *Science* 1997, **276**:795-798.

59. Guo HF, Tong J, Hannan F, Luo L, Zhong Y: **A neurofibromatosis-1-regulated pathway is required for learning in *Drosophila*.** *Nature* 2000, **403**:895-898.

60. Polymenis M, Schmidt EV: **Coupling of cell division to cell growth by translational control of the G_1 cyclin CLN3 in yeast.** *Genes Dev* 1997, **11**:2522-2531.

61. Polymenis M, Schmidt EV: **Coordination of cell growth with cell division.** *Curr Opin Genet Dev* 1999, **9**:76-80.

62. • Galitski T, Saldanha AJ, Styles CA, Lander ES, Fink GR: **Ploidy regulation of gene expression.** *Science* 1999, **285**:251-254.
The experiments described here identify genes that are transcriptionally regulated in response to ploidy in *S. cerevisiae* by means of microarray-based analysis of gene expression. Interestingly, the G_1 cyclins Cln1 and Pcl1 display ploidy-dependent repression. This could explain why cells with higher ploidies divide at a larger size.

63. • Daga RR, Jimenez J: **Translational control of the cdc25 cell cycle phosphatase: a molecular mechanism coupling mitosis to cell growth.** *J Cell Sci* 1999, **112**:3137-3146.
The authors provide evidence that the rate-limiting component of the G_2–M transition in *S. pombe*, Cdc25, is particularly sensitive to reduced translation initiation activity. This effect depends on the 5′ region of the *cdc25* mRNA that is unusually long and contains uORFs, suggesting that Cdc25 might be translationally controlled in a manner similar to the cyclin Cln3 in *S. cerevisiae*. Furthermore, the B-type cyclin Cdc13 is shown to be regulated by a similar mechanism.

64. Sveiczer A, Novak B, Mitchison JM: **The size control of fission yeast revisited.** *J Cell Sci* 1996, **109**:2947-2957.

65. Sveiczer A, Novak B, Mitchison JM: **Mitotic control in the absence of cdc25 mitotic inducer in fission yeast.** *J Cell Sci* 1999, **112**:1085-1092.

66. Martin-Castellanos C, Blanco MA, de Prada JM, Moreno S: **The puc1 cyclin regulates the G_1 phase of the fission yeast cell cycle in response to cell size.** *Mol Biol Cell* 2000, **11**:543-554.

67. Knoblich JA, Sauer K, Jones L, Richardson H, Saint R, Lehner CF: **Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation.** *Cell* 1994, **77**:107-120.

68. Edgar BA, O'Farrell PH: **The three postblastoderm cell cycles of *Drosophila* embryogenesis are regulated in G_2 by string.** *Cell* 1990, **62**:469-480.

69. Neufeld TP, de la Cruz AF, Johnston LA, Edgar BA: **Coordination of growth and cell division in the *Drosophila* wing.** *Cell* 1998, **93**:1183-1193.

70. Edgar BA, Lehman DA, O'Farrell PH: **Transcriptional regulation of *string* (*cdc25*): a link between developmental programming and the cell cycle.** *Development* 1994, **120**:3131-3143.

71. Lehman DA, Patterson B, Johnston LA, Balzer T, Britton JS, Saint R, Edgar BA: **Cis-regulatory elements of the mitotic regulator, *string/Cdc25*.** *Development* 1999, **126**:1793-1803.

72. Richardson HE, O'Keefe LV, Reed SI, Saint R: **A *Drosophila* G_1-specific cyclin E homolog exhibits different modes of expression during embryogenesis.** *Development* 1993, **119**:673-690.

73. Lambertsson A: **The *Minute* genes in *Drosophila* and their molecular functions.** *Adv Genet* 1998, **38**:69-134.

74. Thomas G: **An encore for ribosome biogenesis in the control of cell proliferation.** *Nat Cell Biol* 2000, **2**:E71-E72.

75. Conlon I, Raff M: **Size control in animal development.** *Cell* 1999, **96**:235-244.

76. Stevenson RD, Hill MF, Bryant PJ: **Organ and cell allometry in Hawaiian *Drosophila*: how to make a big fly.** *Proc R Soc Lond* 1995, **259**:105-110.

The insulin receptor signal transduction pathway

Because of its central role in the regulation of glucose homeostasis, the signaling pathway downstream of the insulin receptor (IR) has attracted numerous researchers for several decades. The current literature provides a wealth of information about its components with respect to development, physiology and disease in mammals. In the following sections, I shall try to briefly summarize the current picture of IR signaling. For a more detailed description, the reader is referred to recent review articles (Birnbaum, 2001; Pessin and Saltiel, 2000; Saltiel and Kahn, 2001; Saltiel and Pessin, 2002; Shepherd et al., 1998; Vanhaesebroeck et al., 2001; Virkamaki et al., 1999; White, 2003).

The ligands

The family of insulin-like hormones in mammals comprises at least nine members (ten in humans), with insulin being the most prominent one. Insulin is synthesized in the ß-cells of the islets of Langerhans in the pancreas as pre-pro-insulin and gets subsequently processed (cleavage of signal peptide, removal of C-chain). The mature hormone consists of two polypeptide chains (A- and B-chain) covalently linked by two disulfide bonds. The physiological consequences of insulin action are described below (Insulin signaling and diabetes mellitus). Insulin-like growth factors (IGF-I and IGF-II) are mainly produced by the liver and are key mediators of the growth hormone signal (see IGF signaling downstream of growth hormone). In addition, IGF-I has been shown to exert a neuroprotective function in both the CNS and PNS (Zheng et al., 2000). Insulin-3 (also called Leydig insulin-like hormone) plays an important role during testicular descent by regulating growth and differentiation of the embryonic gubernaculum (Nef and Parada, 1999). Relaxin has been implicated in growth and remodeling of reproductive tissue and has therefore been viewed as a pregnancy hormone (Sherwood et al., 1993), but recent data suggests additional functions for relaxin (Bathgate et al., 2003; Dschietzig and Stangl, 2003; Masterson et al., 2004; Samuel et al., 2004). Another member of the relaxin family (termed H3 relaxin in humans and M3 relaxin in mice, respectively) was identified based on sequence homology (Bathgate et al., 2002). In contrast to relaxin, which is exclusively expressed in the ovary, it is produced in the brain,

suggesting a different (yet to be analyzed) function. The roles of the other insulin-like peptides, termed insulin-4, -5, and -6, remain elusive.
It is conceivable that the action of those ligands can be either enhanced or counteracted by molecules that physically bind the ligand(s) and promote or prevent a productive interaction with the receptor(s). In the case of the IGFs, a family of binding proteins (IGFBPs) that share sequence homologies over the entire length could be identified (Rosenzweig, 2004). Interestingly, IGFBP-7 (also called IGFBP-related protein 1 or Mac25) deviates in its COOH-terminus and was shown to bind IGF with low affinity only. Instead, it is able to bind insulin with high affinity, thereby inhibiting the binding of insulin to its receptor (Yamanaka et al., 1997). Several studies suggest that it can act as a tumor suppressor (Burger et al., 1998; Kato, 2000; Komatsu et al., 2000).

The receptors

The insulin receptor (IR) is a heterotetrameric protein with two α- and two ß-subunits connected via disulfide bonds. Whereas the α-subunits are localized extracellularly and are bound by the ligand(s), the ß-subunits contain a membrane-spanning domain and an intracellular tyrosine kinase domain. Upon ligand binding, the inhibition of the kinase activity of the ß-subunit by the α-subunit gets relieved, which leads to transphosphorylation of the ß-subunits and to the phosphorylation of cellular substrates (see below).
The IGF-I receptor (IGF-IR) and the insulin receptor-related receptor (IRR) belong to the same subfamily of receptor tyrosine kinases and can form functional hybrids. Genetic analyses in mice demonstrated considerable complexity and redundancy in the ligand/receptor interactions (Efstratiadis, 1998). Whereas IGF-I binds exclusively to IGF-IR, IGF-II could be shown to signal through both the IGF-IR and IR (Louvi et al., 1997). Both the IR and the IGF-IR are required for pre- and postnatal growth. Whereas mice lacking IR function die within four days after birth because of severe ketoacidosis (Accili et al., 1996; Joshi et al., 1996), *IGF-IR* mutant mice are reduced in size and die at birth due to respiratory failure (Liu et al., 1993). The growth deficit is aggravated by simultaneous loss of both receptors (Louvi et al., 1997). By contrast, the loss of the orphan receptor IRR, which is highly expressed in pancreatic ß-cells, does not result in developmental or metabolic abnormalities (Kitamura et al.,

2001). Recently, Nef and co-workers discovered an unexpected role for the insulin receptor tyrosine kinase family (Nef et al., 2003). XY mice that are mutant for all three receptors fail to develop male gonads and display a complete female phenotype, probably due to a lack of Sry expression. These findings implicate insulin signaling in male sex determination.

Besides binding to IGF-IR and IR, IGF-II interacts with yet another receptor, IGF-IIR. IGF-IIR displays no homology with receptor tyrosine kinases. It rather corresponds to a mannose 6-phosphate receptor and serves as a sink for IGF-II. Interestingly, IGF-IIR is expressed only from the maternal allele in mice. Mutants inheriting a targeted allele from their mother display an overgrowth phenotype (135% of normal birthweight) and usually die perinatally. The overgrowth correlates with an increased concentration of IGF-II and is completely suppressed by a mutation in either IGF-II or IGF-IR (Ludwig et al., 1996). Mouse embryos lacking both the IGF-IR and the IGF-IIR develop normally (Ludwig et al., 1996).

The receptors for the other insulin-like peptides probably do not belong to the family of receptor tyrosine kinases. Recently, the orphan G-protein coupled receptor LGR7 could be identified to be the physiological receptor of relaxin (Hsu et al., 2002; Krajnc-Franken et al., 2004; Sudo et al., 2003). However, other G-protein coupled receptors were also shown to bind relaxin (Hsu et al., 2002; Liu et al., 2003a; Liu et al., 2003b). Furthermore, insulin-3 signals through the G-protein coupled receptor LGR8/GREAT (Bogatcheva et al., 2003; Ferlin et al., 2003; Kumagai et al., 2002).

The IRS proteins

There is a growing list of intracellular substrates of the insulin/IGF-I receptor tyrosine kinases that link the receptors to signal transduction cascades. Four of the known substrates share sequence homologies and belong to the family of insulin receptor substrate (IRS) proteins (Lee and White, 2004; White and Yenush, 1998; Yenush and White, 1997). IRS family members are characterized by NH2-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains. Binding to the activated receptor occurs through the interaction of a PTB-binding site (the phosphotyrosine motif NPXY) in the juxtamembrane region of the IR/IGF-IR with the PTB domain of IRS. The IRS proteins also possess a number of phosphotyrosine motifs. The phosphorylated tyrosines in the IRS proteins and in other substrates such as Cbl,

Shc, APS and Gab-1 provide docking sites for proteins containing SH2 (Src-homology type 2) domains. The SH2 domain proteins often act as adapter molecules, for instance the p85 regulatory subunit of class 1A phosphatidylinositol-3 kinase (PI3K), or the Grb2 adapter that bridges to the nucleotide exchange factor of the small GTPase Ras. Other SH2 proteins have enzymatic activities like the proteintyrosine phosphatase SHP2 or the non-receptor tyrosine kinase Fyn.
The network of receptors, substrate and adapter molecules is thought to link insulin receptor activity to three main signal transduction pathways: to the PI3K-Akt pathway (see below), the Ras-MAPK pathway (via Grb2/Shc), and the CAP/Cbl pathway that results in the activation of the G protein TC10 (Chiang et al., 2001; Watson et al., 2001).
Knockout analyses for the IRS proteins revealed complementary rather than redundant functions. Whereas mice lacking IRS-1 exhibit a growth retardation phenotype as well as insulin resistance in peripheral tissues (Araki et al., 1994; Tamemoto et al., 1994), IRS-2 knockout mice display insulin resistance both in the periphery and the liver and a decrease in β-cell mass (Kido et al., 2000; Withers et al., 1998), which leads to the development of type 2 diabetes mellitus (see below). In contrast, mice lacking either IRS-3 or IRS-4 do not show severe abnormalities in growth and metabolism (Fantin et al., 2000; Liu et al., 1999).

The second messenger PIP3

The main branch of signaling downstream of the IR/IGF-IR involves the lipid kinase phosphatidylinositol-3-OH kinase (PI3K) (Leevers et al., 1999). Its regulatory subunit (p85) binds via the SH2-domain to phosphotyrosine motifs in IRS proteins, thereby recruiting the catalytic subunit (p110) to the plasma membrane. Alternative ways of PI3K activation include direct binding of p85 to receptor tyrosine kinases and the interaction of p110 with GTP-loaded Ras (Rodriguez-Viciana et al., 1994). Although protein serine kinase activity of PI3K has been reported (Carpenter et al., 1993; Dhand et al., 1994), its principal enzymatic activity consists in the phosphorylation of phosphoinositides at the D3 position (Stephens et al., 1993). Phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) is the major product of PI3K and serves as a second messenger to mediate the signal initiated by IR/IGF-IR activity.

The PIP3 concentration is regulated by the activities of PI3K and of the counteracting lipid phosphatase PTEN (phosphatase and tensin homolog on chromosome 10) (Maehama and Dixon, 1998; Maehama and Dixon, 1999). The loss of PTEN in immortalized mouse embryonic fibroblasts or in embryonic stem cells results in a 2-3 fold increase in the PIP3 concentration (Stambolic et al., 1998; Sun et al., 1999). Interestingly, PTEN is a tumor suppressor that is frequently mutated in a variety of human cancers (Simpson and Parsons, 2001), demonstrating the paramount importance of IR/IGF-I signaling in the regulation of growth. Whether PTEN also possesses protein phosphatase activity is still under debate. At least in vitro, dephosphorylation of focal adhesion kinase (FAK) and Shc by PTEN was demonstrated (Gu et al., 1999; Tamura et al., 1998).

The effectors of PIP3

The best-characterized PIP3 binding motif is the PH domain (Vanhaesebroeck et al., 2001). A variety of signaling proteins contain PH domains and are potentially regulated by the interaction with PIP3 (Leevers et al., 1999). The lipid second messenger PIP3 provides a means to recruit signaling proteins to the plasma membrane, a key step in the regulation of many enzymes. Maybe the best-studied example of activation based on the PIP3-mediated membrane localization is the activation of Akt. Akt is a proto-oncogene composed of an NH2-terminally located PH domain, a central serine-threonine protein kinase domain, and a COOH-terminal regulatory tail. Upon rising PIP3 concentrations, Akt associates with the plasma membrane by virtue of binding to PIP3 via its PH domain. Once at the membrane, Akt is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) at threonine 308 (in Akt1) in the T-loop and by another phosphoinositide-dependent kinase of unknown nature (tentatively called PDK2) at serine 473 near the COOH-terminus. Both phosphorylation events are needed to ensure full Akt kinase activity. Hooking Akt to the membrane results in its constitutive activation (Andjelkovic et al., 1997), demonstrating that (i) the membrane localization is the rate limiting step in the activation of Akt and that (ii) PDK1 (and probably also PDK2) are already localized to the membrane. Oncogenic activation of Akt involves the same mechanism: v-Akt is fused to the viral gag protein (p15, p12, and truncated p30) that is myristoylated at its NH2-terminus and targeted to the plasma membrane. One focus of recent studies

was the identification of Akt substrates. The growing list of proteins that can be phosphorylated by Akt includes the metabolic enzymes GSK3 and 6-phosphofructo-2-kinase; a number of genes involved in survival signaling such as BAD and the Forkhead family transcription factors FKHR, FKHRL1, and AFX; the serine kinase mammalian target of rapamycin (mTOR), endothelial nitric oxide synthase (eNOS), BRCA1, I-κB kinases (IKKs), and Raf (reviewed in (Scheid and Woodgett, 2001)). Consistent with the molecular nature of these substrates, Akt has been primarily implicated in anti-apoptotic signaling (Datta et al., 1999) and in metabolic regulation (Lawlor and Alessi, 2001).

The activating kinase PDK1 also carries a PH domain. Because it displays a higher affinity for PIP3 as compared to that of Akt, PDK1 is supposedly localized to the cell membrane even under nonstimulated conditions. PDK1 phosphorylates AGC kinases including Akt, S6K, SGK, Rsk, PKC isoforms, and PKA. In embryonic stem (ES) cells lacking PDK1 function, phosphorylation of the T-loop motif in Akt, S6K, and Rsk no longer occurred, whereas other AGC family members (namely PKA, MSK1, and AMPK) still were phosphorylated (Williams et al., 2000).

In addition to the kinases Akt and PDK1, a variety of signaling proteins have been postulated to interact with PIP3 via PH domain: Btk family tyrosine kinases, guanine nucleotide exchange factors for the Rho and Arf families of small GTPases, and PLCγ (Leevers et al., 1999). Therefore, raising PIP3 levels is believed to elicit a plethora of cellular responses.

Insulin signaling and diabetes mellitus

In healthy humans, the plasma glucose concentration is kept within the range of 4-7 mM. Insulin is a key player in the metabolic control of glucose homeostasis. Upon high glucose concentration, the islet ß-cells of the pancreas secrete insulin, which promotes the uptake of glucose in muscle and fat cells, stimulates the synthesis of lipids and glycogen, and counteracts lipolysis, glycogenolysis and gluconeogenesis. All these activities contribute to a reduction of the blood glucose level.

Diabetes mellitus is characterized by elevated plasma glucose levels. Three types of diabetes mellitus can be distinguished. Type 1 or insulin-dependent diabetes mellitus (IDDM) results from a deficit in insulin production due to an autoimmune destruction of the ß-cells. It accounts for roughly 10% of the patients. In a subset, the ß-cells are

still present, but they are impaired in glucose-stimulated insulin release because of one of several autosomal dominant mutations that lead to ß-cell dysfunction. This form of diabetes is called maturity onset diabetes of the young (MODY). Most diabetes patients (about 90%) suffer from type 2 or non-insulin dependent diabetes mellitus (NIDDM). The hallmark of type 2 diabetes is the peripheral insulin resistance (i.e. the target tissues such as muscle cells and adipocytes get progressively less responsive to insulin). Initially, the system compensates for the reduced response to insulin by increasing the production of the hormone (hyperinsulinemia), but eventually the ß-cells fail to secrete enough insulin to maintain normal blood glucose concentrations. Type 2 diabetes is a multifactorial disease and shows a strong correlation with obesity.

A series of knockout mice were generated to enhance the understanding of the mechanisms underlying diabetes (Nandi et al., 2004; Terauchi and Kadowaki, 2002). Whereas mice lacking the functions of IR, IRS-2 or Akt-2 did develop a disease related to type 2 diabetes, knockout mice for p85 (a regulatory subunit of PI3K), IRS-1, IRS-3, IRS-4, Akt1, or GLUT4 did not (Saltiel and Kahn, 2001). Diabetes is ameliorated in obese animals by knockouts for the phosphatases PTP1B and SHIP2. The picture got even more complex when tissue-specific knockout mice were analyzed. Surprisingly, mice lacking the IR in the muscle tissue displayed normal glucose tolerance. However, the concentrations of free fatty acids were increased in these animals. Removal of IR function from fat tissue did not affect glucose tolerance, too. Liver-specific IR knockout resulted in impaired glucose tolerance, decreased insulin clearance, and hyperinsulinemia. Mice without IR activity in the islet β-cells exhibited defects in the glucose stimulated insulin secretion. Finally, a brain specific IR knockout yielded a complex phenotype, including increased food intake, mild adiposity, insulin resistance, hypertriglyceridaemia, and reduced fertility (reviewed in (Kitamura et al., 2003; Kulkarni and Okada, 2002; Saltiel and Kahn, 2001)). Thus, the analysis of knockout models demonstrated the existence of a complex regulatory network that governs metabolic control.

IGF signaling downstream of growth hormone

Growth hormone (GH) is produced by the pituitary and is a major regulator of postnatal growth. The somatomedin hypothesis postulates that GH (somatotropin)

induces hepatic expression of a circulating hormone (somatomedin), which in turn elicits responses in the target tissues. There is a large body of evidence for an endocrine role of IGF-I downstream of GH in growth control. First, mice lacking IGF-I are reduced in size (Baker et al., 1993; Liu et al., 1993). Second, circulating IGF-I is diminished in the absence of GH (Lupu et al., 2001). Third, whereas the growth of mice lacking GH can be improved by administration of IGF-I, mice without functional IGF-I remain unresponsive to GH (Liu and LeRoith, 1999). These findings are compatible with the GH-IGF-I axis proposed by the somatomedin hypothesis.
Whereas IGF-I is essential for normal growth throughout development, the role of IGF-II is confined to embryonic growth (DeChiara et al., 1990). Therefore, only IGF-I is a candidate somatomedin. Double mutant mice lacking both GH and IGF-I remain extremely small (only 17% of normal body weight), indicating that the GH-IGF-I axis is a main contributor to growth control (Lupu et al., 2001).
The somatomedin hypothesis, however, is challenged by a series of experimental findings. The phenotype of mice lacking both GH and IGF-I is clearly distinct from that of IGF-I nullizygotes, demonstrating the existence of both independent and overlapping functions (Lupu et al., 2001). Consistently, the expression of IGF-I outside the liver is only in part, if at all, dependent on GH (Lupu et al., 2001). Furthermore, liver-specific conditional knockouts of IGF-I were reported to display a normal growth behavior (Sjogren et al., 1999; Yakar et al., 1999). Despite strongly reduced IGF-I concentrations in the serum, the body size of these mice remained unaffected. However, as the ablation of IGF-I was incomplete in these studies, a growth-promoting role of circulating IGF-I cannot be ruled out.
In order to reconcile the apparently contradictory findings, a four-component model was proposed (Lupu et al., 2001). The endocrine function of hepatic IGF-I (component 1) reflects the overlapping activity of GH and IGF-I. Some of the extrahepatic production of IGF-I also depends on GH (component 2). In addition, both GH and IGF-I exert independent extrahepatic functions (components 3 and 4).

Insulin receptor signaling in model organisms

The signal transduction cascade headed by the insulin receptor has been considerably well conserved during evolution. In the model organism *Caenorhabditis elegans*, insulin receptor signaling regulates development and aging (Finch and

Ruvkun, 2001; Guarente and Kenyon, 2000; Guarente et al., 1998). A large family of 37 insulin-like peptides with largely unknown functions has been discovered (Pierce et al., 2001). An insulin receptor-like tyrosine kinase is encoded by the *daf-2* gene. Interestingly, the requirement for this receptor is non-autonomous and confined to the nervous system (Apfeld and Kenyon, 1998; Wolkow et al., 2000). Signaling downstream of DAF-2 resembles the signaling cascade of mammals: activation of the PI3K AGE-1 results in increasing PIP3 concentrations, which in turn activates PDK-1 and the two Akt-like kinases (AKT-1 and AKT-2). The signal is counteracted by DAF-18, a PTEN ortholog that presumably dephosphorylates PIP3. The Akt kinases phosphorylate and inactivate the Forkhead family transcription factor DAF-16. As *daf-16* mutations are completely epistatic over *daf-2*, *age-1*, and *akt-1*/*akt-2,* the negative regulation of DAF-16 appears to be the crucial outcome of insulin receptor signaling in *C. elegans*.

The exploration of insulin receptor signaling in the fruitfly *Drosophila melanogaster* was initiated by studies on the function of the single insulin receptor ortholog (Chen et al., 1996; Fernandez et al., 1995). Subsequently, Sally Leevers pioneered the growth control research in the Hafen laboratory by an overexpression analysis on PI3K (Leevers et al., 1996). The discovery and characterization of Chico, the fly ortholog of the IRS proteins (Bohni et al., 1999), set the stage for further dissection of this versatile signaling pathway. The work presented here is embedded in this context.

Results

Most data of this thesis have been presented in several publications that will be displayed in the following sections.

A Mosaic Screen to Identify Growth-modulating Genes in *Drosophila melanogaster*

Hugo Stocker, Benno Schindelholz, Sebastian Breuer, and Ernst Hafen

Manuscript

A Mosaic Screen to Identify Growth-regulating Genes in *Drosophila melanogaster*

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ABSTRACT

In order to identify growth-regulating genes, we generated tissue-specific mosaics for newly induced mutations on the right arm of the 3rd chromosome and scored them for size differences in the head and the eyes, the organs where mitotic recombination was forced to occur by the expression of flp recombinase during development. Mutations in genes encoding components of the insulin signaling pathway, namely the insulin receptor (Inr), the lipid kinase PI3K, and the serine/threonine kinase Akt/PKB, were obtained based on a characteristic pinhead phenotype. Two complementation groups yielding very similar phenotypes correspond to the genes encoding the small GTPase Rheb (Ras homolog enriched in brain) and the adaptor molecule Lnk, respectively. Furthermore, alleles of *MASK*, *RhoGAP*, *nicastrin* and an unknown gene (CG2747) were identified due to the resulting head size reduction. Among the mutations resulting in an opposite (bighead) phenotype, we found alleles of the tumor suppressor genes *Tsc1*, *lats*/*warts* and *salvador*. Mutation in the gene that codes for the fly homolog of C-terminal Src kinase (CSK) also fell into this class. We further isolated mutations in *crumbs* and *Hairless* because they significantly increased the size of the mutant eyes. Finally, we report the identification of *Epsin-like protein* (*ELP*) as a candidate locus for a bighead complementation group.

INTRODUCTION

Most of the efforts to unravel the control of growth of multicellular organisms have been spent in elucidating the mechanisms of the cell cycle machinery. Only recently elegant studies in *Drosophila* have reminded us of the predominant role of cellular growth (accumulation of mass) over cell cycle progression (NEUFELD *et al.* 1998; WEIGMANN *et al.* 1997), a fact that had been demonstrated in the single-celled yeast over two decades ago (NURSE 1975). The gene networks governing cellular growth have attracted considerable attention since then. Especially the role of the insulin receptor signaling pathway in the cell-autonomous control of growth during *Drosophila* development has been studied in great detail (GAROFALO 2002; GOBERDHAN and WILSON 2003; STOCKER and HAFEN 2000; WEINKOVE and LEEVERS 2000). Interestingly, larvae lacking the function of the insulin receptor or of one of its downstream signaling components specifically in the eye imaginal disc (the tissue giving rise to the adult eye and the head capsule) will develop small heads and eyes on otherwise normal bodies (BOHNI *et al.* 1999; BROGIOLO *et al.* 2001). These so-called pinhead flies prompted us to exploit the generation of tissue-specific mosaics to systematically screen for mutations that would affect growth either positively (resulting in a pinhead) or negatively (resulting in a big head). The ey-flp system developed by Barry Dickson (NEWSOME *et al.* 2000) turned out to be suitable since it leads to phenotypes that are easy to score and that do not seem to affect the viability of the mosaic flies.

Here we describe our effort to isolate novel growth-affecting mutations on the right arm of the 3rd chromosome. We report the isolation of ten growth promoting and eight growth inhibiting complementation groups.

MATERIALS AND METHODS

Fly stocks

To generate tissue-specific mosaics, we made use of the ey-flp system described in (NEWSOME *et al.* 2000). The deficiencies used in the mapping procedure are listed in Tables 1 and 2. The following mutations were used for complementation analyses: *Inr*^{5545} (FERNANDEZ *et al.* 1995), *Dp110A* (WEINKOVE *et al.* 1999), *dAkt*^{1} (STAVELEY *et al.* 1998), *crb*^{2}, *H*^{1}*, Tsc1* (TAPON *et al.* 2001), and *lats* (XU *et al.* 1995).

Screening of mosaic flies

Males carrying FRT sites on the right arm of chromosome 3 were fed with 33mM EMS according to standard protocols and crossed to females of the genotype *y w ey-flp*; *FRT82 w*$^{+}$ *cl3R3*/*TM6B y*$^{+}$. *cl3R3* is a recessive cell-lethal mutation that has been generated on the *FRT82 w*$^{+}$ chromosome (NEWSOME *et al.* 2000). Half of the F1 progeny was of the genotype *y w ey-flp/+* or Y; *FRT82 */FRT82 w*$^{+}$ *cl3R3* and was scored for eyes and heads of abnormal size. Positives were re-crossed to *y w ey-flp*; *FRT82 w*$^{+}$ *cl3R3*/*TM6B y*$^{+}$ to check for germline transmission. Of the 50000 flies screened approximately 1000 showed size-defects of the head, whereof 390 stable lines could be established.

Mapping of the mutations

For each complementation group, one representative allele was mapped meiotically based on the phenotype. The markers used were: *mini-w*$^{+}$ (cytological position 87E), *w*$^{+}$ (90E), and *y*$^{+}$ (96E). The rough map position was confirmed and refined by complementation analysis using deficiencies (where available). Fine-mapping was done by assessing the frequency of recombination between alleles and nearby P-element insertions. The P-elements used for the mapping are listed in Tables 1 and 2.

RESULTS

Mutations in components of the insulin receptor signaling pathway impair cellular growth in a cell-autonomous fashion (BOHNI *et al.* 1999; BROGIOLO *et al.* 2001; VERDU *et al.* 1999; WEINKOVE *et al.* 1999). Clones of mutant cells bear a severe growth disadvantage and remain small compared to their wild-type sister clones. If mitotic recombination is forced to occur by the constant supply of flp recombinase and the sister clone gets eliminated by means of a cell-lethal mutation, these clones can, however, cover substantial fractions of whole organs. Driving the expression of the flp recombinase under the control of *eyeless* regulatory sequences results in the formation of tissue-specific clones in the eye imaginal disc solely. In combination with a cell-lethal mutation on the homologous chromosome, this system allows to generate eyes and head capsules largely composed of cells that are homozygous mutant for the gene of interest (NEWSOME *et al.* 2000). Such mosaic flies that lack the function of the IRS homolog Chico or of the insulin receptor (Inr) specifically in the descendants of the eye imaginal disc show a very characteristic phenotype. While their bodies are of normal size, their eyes and heads are dramatically reduced (BOHNI *et al.* 1999; BROGIOLO *et al.* 2001). We wished to identify mutations in growth-modulating genes based on similar phenotypes. To this end, males carrying target sites for the flp recombinase near the base of the right arm of the 3rd chromosome (FRT82) were subjected to EMS mutagenesis and crossed to females that brought in four elements: the source of the recombinase (*ey-flp*), FRT sites at the corresponding position, a dominant eye marker (w^+) and a cell-lethal mutation (*cl3R3*). In the mosaic flies of the F1 generation, the effects of homozygosity for newly induced mutations on 3R could be observed in the heads and the eyes. The presence of homozygous mutant tissue could easily be visualized by the loss of the pigment marker w^+ resulting in white eye tissue.

We scored 50000 mosaic flies for size defects and recovered 321 mutations giving rise to smaller eyes and 69 mutations leading to bigger eyes. The mutations were further classified based on whether they primarily affect the number or the size of individual ommatidial units, or both (Figure 1). Subsequently, extensive complementation analyses were initiated within these classes, and a total of 18 complementation groups could be established.

Class S1: Reduction of both cell number and cell size

Ey-flp clones for mutations in *Inr* or in *chico* cause a pinhead phenotype due to fewer and smaller cells. The differentiation of various cell types, however, is not affected. Therefore, albeit much reduced in size, such eyes have a normal appearance and a smooth surface. We classified all phenotypes with similar characteristics as S1. Eyes that also showed a reduction in both cell number and cell size but displayed a rough eye surface were scored as S2 mutations. The roughness could be due to the mutation responsible for the size defect. Alternatively, it could be caused by an unrelated second-site mutation. Since we could not *a priori* distinguish between these possibilities, we treated the S1 and S2 classes similarly.

We could establish six complementation groups in the S1/S2 class (Table 1, Figure 2B-E). As the genes encoding the three core components of the Inr signaling cascade (Inr, Dp110/PI3K, Akt/PKB) all map to 3R, we tested whether we isolated novel alleles by complementation analysis with already existing alleles. Indeed, we found 48 alleles of *Inr*, 19 alleles of *Dp110/PI3K* and 13 alleles of *Akt/PKB*. These high numbers of alleles argue that the screen was very efficient and that saturation was probably reached.

S1A is comprised of 10 alleles that gave rise to very similar pinhead phenotypes as mutations in Inr signaling components. It was meiotically mapped close to the centromere, and subsequent SNP mapping lead to the identification of *Rheb* as the affected gene (STOCKER *et al.* 2003).

S1B, a large complementation group consisting of 26 alleles, was roughly mapped to the cytological region 95. Using deficiencies with breakpoints in this interval, the candidate region could be further refined to 95D11 – 95F7. We tested P-element insertions in 95D – 95F and found that line *l(3)S005343* (DEAK *et al.* 1997) fails to complement two representative alleles of S1B. By remobilizing the P-element in *l(3)S005343* we could confirm a causal relationship between the insertion and the non-complementation with S1B (30 out of 38 w^- revertants did complement S1B). Interestingly, two alleles of S1B that showed a less pronounced pinhead phenotype in ey-flp clones survived in trans to *l(3)S005343*, but the resulting adults were clearly reduced in size. The insertion site of *l(3)S005343* was determined by the plasmid rescue technique. The P-element lies 2 kb upstream of the gene encoding MASK (Multiple ankyrin-repeats, single KH domain) that has recently been identified in a screen for enhancers of a phenotype caused by a dominant negative form of the

phosphatase Corkscrew (SMITH *et al.* 2002). MASK encodes a large protein with up to 23 ankyrin repeats, two putative nuclear localization signals (NLS) and a KH domain.

The four alleles of complementation group S1F are very special in that they give rise to small adults in all heteroallelic combinations. The cytological map position of S1F based on phenotypic meiotic mapping is 96D – 96F. S1F alleles fail to complement a P-element insertion in the gene encoding Lnk, an adaptor protein with a PH- and an SH2-domain.

Class S3: Reduction of cell size

In contrast to the S1 class, pinheads of the S3 class contain a roughly normal number of ommatidia. The size defect, therefore, is primarily due to a reduction in cell size (provided that the ommatidial units are properly built). We could establish three complementation groups belonging to class S3 (Table 1, Figure 2F-H). S3A is represented by seven alleles and could be localized to the numbered divisions 84/85 by phenotypic meiotic mapping. As this region has been subjected to extensive genetic analyses, a number of deficiencies with well-defined breakpoints allowed a subdivision of the interval defined by the genes *doublesex* and *puckered*. S3A could be placed to 84E8-12 because of non-complementation with *Df(3R)dsx21* and complementation with *Df(3R)dsx15* and *Df(3R)dsx43*. In addition, S3A alleles failed to complement *l(3)84Ee* that had been isolated in a screen for lethal mutations in 84D-E. Complementation group *l(3)84Ee* is represented by a single allele that was isolated among 2386 lines (BAKER *et al.* 1991). The number of alleles from both screens point to a rather small gene. However, we were able to identify molecular lesions in CG2747 that encodes a huge protein of unknown function and without any discernible motif (two isoforms of 2165 and 2117 amino acids, respectively). It remains to be determined whether the lesions found in CG2747 are causally related to the pinhead phenotype of S3A.

S3B and S3C consist of ten alleles each. Classical meiotic mapping and subsequent fine mapping enabled us to narrow down the candidate regions to 84D-E and 88C-E, respectively (Table 1). The candidate region for S3C could be further refined by SNP mapping, and subsequently mutations in *RhoGAP88C* were identified. The gene affected by the S3B alleles remains to be determined.

Class S4: Reduction of cell number

A small number of pinheads were characterized by the dramatically reduced number of ommatidia that appeared to be of normal size. Most of these mutations could not be classified into complementation groups. Two mutations, however, failed to complement each other as well as two mutations of the Ras-like class. Characteristic of Ras-like mutations is a pinhead phenotype without homozygous mutant tissue in ey-flp clones. This phenotype emerges because the mutant cells are able to progress through the cell cycle (albeit at a reduced rate) but are bound to die as soon as they should initiate differentiation into the different cell types (HALFAR *et al.* 2001). It is conceivable that mutations of the S4 class represent hypomorphic alleles of the Ras-like class. The S4A complementation group could be mapped to the letter division 96B. SNP mapping allowed identifying *nicastrin* as the affected gene (NAIRZ *et al.* 2002).

Taken together, 153 of the 321 pinhead mutations could be assigned to ten complementation groups. The genes associated with nine of these complementation groups could be determined. Whereas a growth-promoting role of *Inr*, *Dp110/PI3K* and *Akt/PKB* has already been shown, we identified MASK, Rheb and Lnk as novel growth regulators.

In addition to the pinhead mutations, our screen yielded a total of 69 mutations that resulted in increased size of the eyes and the head. We applied analogous criteria to subdivide these mutations into big head classes (Table 2).

Class B1: Increase in cell number and cell size

The mutations resulting in an increased eye field due to more and slightly bigger ommatidia defined three complementation groups. Unexpectedly, we found that two of the complementation groups correspond to the previously described genes *crumbs* and *Hairless*, respectively (Figure 3B,C). *crumbs* encodes a transmembrane protein with EGF- and laminin A-repeats which plays a role in the biogenesis of adherens junctions and in the maintenance of apical membrane identity (GRAWE *et al.* 1996; TEPASS 1996; TEPASS *et al.* 1990; WODARZ *et al.* 1995). The relatively high number of *crumbs* alleles isolated in our screen (13) is consistent with the large size of the

coding region (Crumbs consists of 2139 amino acids). Three alleles of the B1 class failed to complement *Hairless* that encodes a negative component of the Notch signal transduction cascade (BANG *et al.* 1991; BANG and POSAKONY 1992; MAIER *et al.* 1992). Hairless has been shown to bind to Suppressor of Hairless (Su(H)) and thereby antagonizing its DNA-binding activity (BROU *et al.* 1994). The haplo-insufficient phenotype of *Hairless* (loss of the humeral bristles) is also evident in the newly isolated alleles.
Another complementation group that also encompasses three alleles (B1E) was only recently identified and was mapped to the cytological region 87F-88B.

Class B3: Increase in cell size

12 mutations displayed a dramatic increase in cell size, whereas the number of ommatidia appeared to be only slightly increased (Figure 3D). This phenotype is very reminiscent of the consequences of loss of PTEN function (GAO *et al.* 2000; GOBERDHAN *et al.* 1999; HUANG *et al.* 1999). The tumor suppressor PTEN is a lipid phosphatase that antagonizes the activity of PI3K. Therefore, the mutants of the B3 class are likely to code for negative regulators of Inr signaling. We found that all 12 mutations belong to a single complementation group that maps to 95D11 – 95F15. An obvious candidate gene, the fly homologue of the tuberous sclerosis gene *Tsc1*, lies in 95E4-5 (ITO and RUBIN 1999). Recently, three groups reported that mutations in *Tsc1* result in phenotypes indistinguishable from those observed in our screen (GAO and PAN 2001; POTTER *et al.* 2001; TAPON *et al.* 2001). In fact, complementation analysis confirmed that B3 corresponds to *Tsc1*.

Class B4: Increase in cell number

Mutations belonging to four complementation groups resulted in a hyperproliferative phenotype. The supernumerary ommatidia caused the formation of folds that gave the eyes a tumerous appearance (Figure 3E-H). The four alleles of complementation group B4B could be correlated with the tumor suppressor gene *lats/warts* (JUSTICE *et al.* 1995; XU *et al.* 1995). *lats/warts* encodes a serine/threonine kinase that has been implicated in the regulation of cdc2 (TAO *et al.* 1999).
The localization of the largest complementation group of the B4 class (B4A, nine alleles) could be narrowed down to the interval 94D1 – 94D10-13, which corresponds

to about 150 kb. We were able to detect lesions in CG13831 that corresponds to the recently identified *salvador* gene (KANGO-SINGH *et al.* 2002; TAPON *et al.* 2002). *salvador* encodes a WW-domain protein that binds to Lats/Warts (TAPON *et al.* 2002). Complementation group B4C consists of three alleles and could be placed to 86E6 – 86E19 by meiotic mapping followed by complementation with deficiencies. We identified three lethal P-element insertions in this interval that failed to complement B4C. The analysis of the insertion sites by plasmid rescue revealed that all three P-elements reside within gene CG17309. There exist two classes of ESTs for CG17309 that result from the usage of two different upstream exons. All P-insertions lie in the first intron with respect to the more abundant transcript and upstream of the transcriptional start site of the rarer transcript, respectively. The protein encoded by CG17309 shares extensive homologies to C-terminal Src kinase (CSK) in its C-terminal half (SH2 domain and tyrosine kinase domain), but its N-terminus lacks any homologies to other proteins in the database.
The four alleles of B4D map to 94A in close proximity to the P-element insertion *l(3)L3560*. Using the SNP mapping technique, we identified mutations in CG31170. CG31170 codes for a protein of 633 amino acids with an N-terminal ENTH (epsin N-terminal homology) domain. Therefore, we named CG31170 Epsin-like protein (ELP).

In summary, 52 of the 69 big head mutations fall into eight complementation groups. Interestingly, we could identify mutations in two genes, *crumbs* and *Hairless*, which had been studied in great detail before without revealing a role in growth control. The isolation of alleles of the tumor suppressor genes *lats/warts* and *dTsc1* is less surprising. Furthermore, we identified a novel protein with strong homologies to CSK. The gene mutated in the bighead complementation group B1E remains to be determined.

DISCUSSION

Growth is a central aspect of life, and its regulation and coordination poses a major problem to all multicellular organisms. To gain insight into the signaling networks governing growth, it is of outstanding importance to discover the genes involved in growth control. This can be achieved by identifying mutations that will affect proper growth control in genetically amenable model systems. We wished to determine all genes involved in the regulation of growth in *Drosophila melanogaster* by screening for mutations affecting the size of particular organs, the head and the eyes. This study describes the growth-modulating mutations that we identified on the right arm of the 3rd chromosome.

Screening for growth mutation faces two major limitations. First, most mutations are expected to be strictly recessive, i.e. the loss of one copy will not alter the size of the organism. In order to check the consequences of homozygosity for such mutations, a F2 screen would be required. The amount of work associated with an F2 screen, however, would not allow screening the genome to saturation. Second, it is conceivable that the complete loss of growth-modulating genes might result in lethality, thereby excluding these genes from detection in an F2 screen. To circumvent these problems, we decided to generate tissue-specific mosaics. The ey-flp technique (NEWSOME *et al.* 2000) allowed us to screen flies with largely homozygous heads on otherwise heterozygous bodies in the F1 generation. The proportions of the mutant eyes and head relative to the normal-sized thorax served as indicator for growth defects. Since the fly head is dispensable for viability, we could even detect mutations that cause lethality in the homozygous state.

The mutations we found could affect growth in many ways. They might (a) interfere with the patterning of the organ, (b) directly regulate cellular growth (= accumulation of biomass), (c) influence the survival of the mutant cells or (d) any combination of these possibilities. Our primary interest was directed to the class of mutations that exclusively impinge on cellular growth. To avoid patterning or apoptosis mutants, we decided to selectively screen for flies with eyes of abnormal size but rather normal morphology. We discarded all flies that either showed morphological anomalies of the eyes (altered shape, scars, loss of ommatidial structure etc) or that did not carry homozygous mutant cells (some exceptions will be discussed below).

The observed size differences could be due to changes in cell size, cell number, or both. We used these criteria to subdivide the growth mutations into several phenotypic classes (Table 1). How do the different phenotypes arise? Previous studies have shown that impaired cellular growth caused by a deficit in Inr signaling results in a reduction of both cell number and cell size without affecting patterning. Therefore, we would expect all mutations impairing Inr signaling strength to fall into class S1. Interestingly, mutations in the gene encoding the *Drosophila* homologue of ribosomal S6 kinase (p70 S6K) give rise to adult flies that are reduced in size due to smaller cells solely (MONTAGNE *et al.* 1999). It is unclear, however, whether such a mutation would give rise to a S3 pinhead. In the case of *dS6K*, tissue-specific mosaics failed to produce pinheads, probably as a consequence of the stability of the protein (J. Montagne, personal communication). Alternatively, mutations in the S3 class could reflect a primary defect in cell size measurement. This would imply that the organ size is determined relative to the cell size (otherwise the organ would reach a normal size by adding more cells). An opposite phenotype is observed in class S4 (mutant eyes that consist of fewer cells of apparently normal size). This defect cannot simply be caused by blocking cell cycle progression, because in such a case the heterozygous cells that did not undergo mitotic recombination would compensate for the slowly dividing homozygous mutant cells and take over the majority of the eye tissue. The phenotype could arise as a consequence of a failure of the mutant cells to differentiate (comparable to the situation in clones of cells mutant for *Ras*, (HALFAR *et al.* 2001)) or as a result of a block in the eye development program (e.g. disturbed progression of the morphogenetic furrow). Since we tried to exclude mutants affecting eye development, we only kept the lines that showed a clearly reduced head size indicative of growth deficits.

The bighead mutants were classified accordingly. In contrast to mutations in positively acting components of the Inr signaling cascade, tissue-specific mosaics for a negatively acting component, the tumor suppressor gene *PTEN*, primarily affect cell size but only moderately increase cell number. Mutations in genes counteracting Inr signaling can therefore be expected to fall into class B3. Bigheads with more (and slightly larger) ommatidia (class B1) could arise from an aberrant definition of the eye field. Even if they were not a direct consequence of stimulated cellular growth, they might tell us a lot about how the size of an organ gets determined. Finally, bigheads of class B4 were chosen despite the fact that they display abnormal eye morphology

because the phenotype was suggestive of massive overproliferation (and therefore overgrowth).

Ordering the 390 growth mutations into complementation groups represented a major task. The superficial classification of the mutations upon visual inspection proved to be very helpful for the complementation analyses. First, we tested the strongest mutations within each class against each other. Second, a representative member of every complementation group was crossed to the whole collection of growth mutations. This procedure yielded ten complementation groups in the pinhead class and nine complementation groups in the big head class.

To map the mutations, we applied the following strategy: (1) The mutations were mapped relative to three dominant markers based on the phenotype. This quick method provided us with a rather rough map position. Since the mapping relied on the phenotype and not on other criteria such as lethality, we could avoid problems with background mutations. Furthermore, composite phenotypes (e.g. rough pinheads caused by more that one mutation on 3R) became immediately evident. (2) We made use of deficiencies to confirm and to further refine the map position. (3) Nearby P-element insertions were chosen for the fine mapping. The distance between the mutation and the dominant marker of the P-element was determined by the frequency of recombinant chromosomes having lost both the dominant marker and the mutation (as assessed by the loss of lethality over another allele of the same complementation group). (4) The closest P-elements are selected for SNP mapping. This technique makes use of normally occurring single nucleotide polymorphisms between different strains that serve as molecular markers to determine the sites of recombination very precisely. SNPs in fragments of up to 1000 bp can easily be detected by duplex HPLC (DHPLC) that exploits the changes in the melting behavior of DNA caused by mismatches (NAIRZ *et al.* 2002).

We were able to clarify the identity of nine growth promoting and of seven growth inhibiting genes so far. As expected, mutations in components of the Inr signal transduction pathway turned up in the S1 class. In total, we isolated 48 novel *Inr* alleles, 19 mutations in the gene encoding the catalytic subunit of PI3K (*Dp110*) and 13 alleles of *Akt/PKB*. These numbers show a clear correlation with the length of the corresponding proteins (Inr: 2146 amino acids, Dp110/PI3K: 1088 amino acids, Akt/PKB: 611 amino acids). Furthermore, in the light of the high number of alleles we feel confident that the right arm of the 3rd chromosome has been screened to saturation.

Mutations in all the three central components of Inr signaling have been described before. Whereas a number of *Inr* alleles have already been analyzed (BROGIOLO *et al.* 2001; CHEN *et al.* 1996; FERNANDEZ *et al.* 1995), the only available loss-of-function mutation of *Dp110/PI3K* is a synthetic null allele (a small deficiency combined with a genomic rescue construct for the neighboring gene, *Hairless*)(WEINKOVE *et al.* 1999). It will therefore be interesting to determine the molecular lesions in the novel *Dp110/PI 3K* alleles and to correlate them with the phenotypic strength. One special allele (5W3) should provide us with a particularly useful tool for further studies on growth regulation and other aspects of Inr signaling because it represents a temperature-sensitive mutation. While it is fully viable over a deficiency at 18°C, it behaves like a strong loss-of-function allele at 25 °C. Temperature-shift experiments have already revealed that the female sterility associated with Inr signaling mutations is reversible (data not shown). Two mutations in *Akt/PKB* have been reported previously, but the data concerning the phenotypic analysis have remained somewhat confusing (SCANGA *et al.* 2000; STAVELEY *et al.* 1998; VERDU *et al.* 1999). In contrast to *Inr* and *chico*, mutants in *Akt/PKB* were claimed to affect cell size solely. In a study presented elsewhere, we clearly demonstrate that flies homozygous mutant for a hypomorphic allele of *Akt/PKB* are reduced in size owing to fewer and smaller cells. In the screen described here, we isolated at least six additional hypomorphic alleles of *Akt/PKB* that were viable in combination with the null allele $dAkt^{1}$. All these combinations resulted in small flies with a significant reduction in both cell size and cell number (data not shown). Three additional complementation groups belonging to class S1 were established. It is conceivable that the affected gene products also play a role in Inr signaling. In fact, S1A and S1F turned out to correspond to *Rheb* and *Lnk*, respectively. The characterization of *Rheb* was described elsewhere (STOCKER *et al.* 2003). It encodes a small GTPase of the Ras superfamily. Epistasis analysis placed it in the TOR branch downstream of Inr signaling, between the Tsc1/2 complex and TOR. Several studies demonstrated that Tsc2 acts as the GTPase activating protein (GAP) of Rheb, thereby establishing a direct link from the Tsc1/2 tumor suppressor complex to Rheb (GARAMI *et al.* 2003; INOKI *et al.* 2003; TEE *et al.* 2003; ZHANG *et al.* 2003). Lnk is the only *Drosophila* homolog of three members of a family of adaptor molecules, consisting of Lnk (HUANG *et al.* 1995), APS (YOKOUCHI *et al.* 1997) and SH2-B (KOTANI *et al.* 1998). The hallmark of these family members is the presence of a pleckstrin homology (PH) domain, a Src homology type 2 (SH2) domain, and

tyrosine phosphorylation sites that form Grb2 docking sites. APS and SH2-B directly interact with and are phosphorylated by the insulin receptor (AHMED and PILLAY 2001; AHMED *et al.* 1999; AHMED *et al.* 2000; HU *et al.* 2003; KOTANI *et al.* 1998). Knockout mice have been generated to get insight into the function of the three family members. Whereas mice lacking Lnk are viable and show relatively mild defects in B cell regulation (TAKAKI *et al.* 2000), *APS*$^{-/-}$ animals display increased insulin sensitivity (MINAMI *et al.* 2003). Mice devoid of SH2-B are slightly growth-impaired and have underdeveloped genital organs (OHTSUKA *et al.* 2002). The phenotypes of double and triple knockout mice remain to be determined. In light of the biochemical data on the mouse homologs, we assume that *Drosophila* Lnk bridges between the Inr and downstream effectors such as PI3K and, possibly, Drk/Grb2. Therefore, Lnk could play a role that is partially redundant with the function of the IRS homolog Chico. In fact, whereas *chico* and *Lnk* single mutants are viable and reduced in size, *chico Lnk* double mutants are lethal. This could explain why chico is the only non-essential core component of Inr signaling described so far. Another possibility is that Lnk mediates Inr signaling via Drk-Ras. Whereas the Inr loss-of-function phenotype did not reveal any sign of reduced Ras-MAPK activity, overexpression of an activated Inr resulted in MAPK phosphorylation (BROGIOLO *et al.* 2001). Whether this crosstalk requires Lnk function needs to be investigated.

The screen revealed a growth promoting function for MASK. The presumptive gene product carries up to 23 ankyrin repeats (depending on the prediction program and the stringency used), two putative nuclear localization signals and a well-conserved KH-domain. Whereas ankyrin repeats have been shown to form reactive surfaces that primarily mediate protein-protein interactions (SEDGWICK and SMERDON 1999), the KH domain is supposed to bind RNA (SIOMI *et al.* 1994). It was first identified in the hnRNP K protein (KH = hnRNP K homology). The *Drosophila* genome encodes 27 proteins with KH domains, whereof 18 proteins possess a single KH domain (LASKO 2000). They fulfill a variety of cellular functions ranging from prevention of nuclear export of a specific mRNA (NABEL-ROSEN *et al.* 1999) to the intracellular localization of mRNAs (DESHLER *et al.* 1998; HAVIN *et al.* 1998; ROSS *et al.* 1997). A prominent member of the KH domain protein family, the human fragile-X associated protein (FMRP), also has a homologue in *Drosophila* (CG6203; (LASKO 2000)) and is thought to shuttle between the nucleus and the cytoplasm (FENG *et al.* 1997b). Its association with large mRNP particles and ribosomes is suggestive of a role in

translational control (CEMAN *et al.* 1999; FENG *et al.* 1997a). The *Drosophila* P-element somatic inhibitor (PSI), a protein with four KH domains, is a member of the RNA-binding protein complex that mediates the tissue-specific alternative splicing of the P-element transposase mRNA. Interestingly, mutations in *bancal* that encodes a *Drosophila* homologue of hnRNP K lead to a reduction in appendage size owing to fewer cells (CHARROUX *et al.* 1999). High levels of *bancal* expression, on the other hand, result in programmed cell death (CHARROUX *et al.* 1999). Thus, correct levels of *bancal* expression seem to be crucial for normal development. Consistent with this notion, Bancal appears to negatively regulate its own expression (CHARROUX *et al.* 1999). The variety of functions exerted by KH-domain containing proteins renders functional predictions difficult. Recently, MASK has been isolated as a putative component of Ras-MAPK signaling (SMITH *et al.* 2002). The function of MASK, however, remains elusive.

Among the mutations in growth inhibiting genes, we were most interested in the B3 class because the phenotypes associated with these mutants are very similar to those obtained with *PTEN* alleles. We therefore speculate that B3 alleles represent mutations in genes counteracting Inr signaling. All 12 alleles of class B3 formed a single complementation group, indicating that there is only one locus on 3R that genetically behaves like *PTEN*. Our mapping results pointed to the *Drosophila* homologue of the tumor suppressor gene *Tsc1* as the likely candidate because mutations in *gigas* that encodes the homologue of Tsc2 display similar phenotypes (ITO and RUBIN 1999). Indeed, in the course of our work three other groups reported that mutations in *Tsc1* result in growth phenotypes comparable to *PTEN* mutants, and that they genetically interact with mutations in the Inr signaling cascade (GAO and PAN 2001; POTTER *et al.* 2001; TAPON *et al.* 2001). Tuberous sclerosis complex (TSC) is a dominant disorder characterized by the occurrence of hamartomas in many organs (reviewed in CHEADLE *et al.* 2000). These hamartomas are growths consisting of differentiated but disorganized cells, and they frequently contain giant cells. A causal relationship between inherited TSC and mutations in two genes, *TSC1* and *TSC2*, has been demonstrated (VAN SLEGTENHORST *et al.* 1997). *TSC1* encodes a protein of 1164 amino acids, hamartin, containing two putative coiled-coil domains near its C-terminus. *TSC2* codes for an 1807 amino acid protein called tuberin that also contains predicted coiled-coil domains as well as a putative GTPase-activating protein (GAP) domain. The two proteins have been shown to

interact physically via their coiled-coil domains (NELLIST *et al.* 1999; PLANK *et al.* 1998; VAN SLEGTENHORST *et al.* 1998). Mutations in either gene result in the same clinical manifestations, presumably because they disrupt the formation of a functional complex. All the 152 reported *TSC1* mutations from human patients as well as the four mutations in the fly homologue of *TSC1* lead to a truncation of the protein. It will be interesting to determine whether other kinds of mutations can be found among the 12 alleles isolated in our screen. Genetic analysis has revealed a function of the TSC complex in the negative regulation of TOR (GAO *et al.* 2002; RADIMERSKI *et al.* 2002). Consistently, the target of the GAP activity of Tsc2, the small GTPase Rheb, acts upstream of TOR.

The two genes that we found to be mutated in the complementation groups B1A and B1D were less expected. *crumbs* codes for a transmembrane protein with a huge extracellular domain containing 30 EGF- and four laminin A-type repeats, respectively. It has been shown to exert a crucial function in the formation of adherens junctions and in the establishment of apical polarity of epithelial cells (GRAWE *et al.* 1996; WODARZ *et al.* 1995). Loss of Crumbs function results in complete degeneration of all ectodermally-derived epithelia during embryogenesis. Crumbs forms complexes with the PDZ-domain protein Discs lost (Dlt) (BHAT *et al.* 1999). A function for Crumbs in imaginal disc development has not been described yet.

Hairless encodes a protein that antagonizes the DNA-binding activity of Suppressor of Hairless and thereby counteracts Notch signaling. It is known that the function of Hairless is required only in a subset of Notch-mediated developmental decisions, especially in the development of the peripheral nervous system (SCHWEISGUTH and LECOURTOIS 1998). Recently, a growth-promoting role for Notch signaling during eye development has been suggested. It appears that Notch activity is required along the equator to define the size of the eye field, which in turn triggers the proliferation of undifferentiated cells of the eye primordium (CHAO *et al.* 2004; CHO and CHOI 1998; DOMINGUEZ and DE CELIS 1998; KENYON *et al.* 2003; PAPAYANNOPOULOS *et al.* 1998). If Hairless acted in this process to restrict Notch activity, loss of Hairless function might result in an expansion of the eye field, which could account for the big head phenotype. A similar role for Notch signaling in organ growth control has also been observed in the leg imaginal disc (DE CELIS *et al.* 1998; RAUSKOLB and IRVINE 1999). It is tempting to speculate that in *crumbs* mutants, Notch signaling gets somehow

intensified. It will be interesting to identify the remaining gene of the same class B1 and to test whether it also modulates Notch signaling strength.

Mutants of class B4 are special in that they lose the normal epithelial organization of the eyes. They tend to form huge folds that are reminiscent of tumors. Since they obviously cause massive overgrowth, we decided to further characterize these mutants although they do not meet all our criteria. Complementation group B4B turned out to correspond to the tumor suppressor gene *lats/warts*. This gene had previously been identified based on the phenotype in clones of mutant cells (apical hypertrophy of imaginal epithelial cells, (JUSTICE *et al.* 1995; XU *et al.* 1995)). It encodes a serine/threonine kinase related to cAMP-dependent kinases. Mammalian homologues of Lats/Warts have also been reported (HORI *et al.* 2000; TAO *et al.* 1999; YABUTA *et al.* 2000). Human Lats1 was shown to complex with Cdc2 early in mitosis, thereby excluding the binding of CyclinA and inhibiting the kinase activity of Cdc2 which results in G2/M arrest (TAO *et al.* 1999; XIA *et al.* 2002; YANG *et al.* 2001). Besides other defects in the endocrine system, *lats1* knockout mice develop soft-tissue sarcomas and are highly sensitive to carcinogenic treatments (ST JOHN *et al.* 1999). Mouse embryos lacking Kpm/Lats2 exhibit overgrowth in restricted tissues, and *lats2*$^{-/-}$ MEFs acquire a growth advantage and display genomic instability (MCPHERSON *et al.* 2004). The tumor suppressor gene *lats/warts*, therefore, exemplifies the value of the model system *Drosophila* to discover growth-regulating genes that are also relevant to tumorigenesis in humans.

Our screen yielded a novel tumor suppressor gene coding for a protein that shares strong homologies with C-terminal Src kinase (CSK) in its C-terminal half. The protein carries highly conserved SH2 and tyrosine kinase domains, but no SH3 domain. Since the N-terminal half does not show significant homologies to other proteins, it is likely that the regulation of the tyrosine kinase activity is achieved in a way different to the regulation of Src by CSK. The similarity of the phenotypes suggests that Lats/Warts and the novel tyrosine kinase might act in the same process(es). Indeed, Stewart and co-workers identified *CSK* mutations as dominant modifiers of lats-dependent phenotypes (STEWART *et al.* 2003). CSK is able to phosphorylate Lats at a conserved C-terminal tyrosine, and this phosphorylation is required for normal Lats function (STEWART *et al.* 2003).

The complementation group displaying the strongest overproliferation phenotype, B4A, corresponds to the *salvador* gene. Salvador, a WW-domain containing protein, restricts proliferation and promotes apoptosis (KANGO-SINGH *et al.* 2002; TAPON *et al.*

2002). These effects are, at least in part, mediated by the regulation of Cyclin E and DIAP1 expression, respectively (TAPON *et al.* 2002). Interestingly, Salvador is evolutionarily conserved, and mutations in the human ortholog, hWW45, were discovered in three cancer cell lines (TAPON *et al.* 2002). Recently, the serine/threonine kinase Hippo, the ortholog of human MST1 and MST2 kinases, was found as a novel member of the Hippo-Salvador-Warts signaling cassette (HARVEY *et al.* 2003; PANTALACCI *et al.* 2003; UDAN *et al.* 2003; WU *et al.* 2003). Hippo binds to and phosphorylates Salvador, thereby forming a physical and functional complex with Salvador and Warts (WU *et al.* 2003). It is also evolutionarily conserved, since the human ortholog is capable of rescuing the *Drosophila* mutation (WU *et al.* 2003). Therefore, it is tempting to speculate that the Hippo-Salvador-Warts complex might act as a tumor suppressor in humans.

Concluding remarks

We attempted to identify all growth-modulating genes on the right arm of chromosome 3 by screening tissue-specific mosaics for size abnormalities. We ended up with a reasonable number of complementation groups for both the growth stimulating and growth inhibiting genes. The identification of the affected genes has provided evidence for both the specificity of the screen and the relevance of the mutants that were found. Thus, we feel confident that our strategy to search for growth-modulating mutations in the model organism *Drosophila* is a valuable tool to identify and characterize novel genes that will hopefully contribute to our understanding of human cancer.

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FIGURE LEGENDS

Figure 1 Schematic representation of phenotypic classes.

All the growth-modulating alleles have been classified according to their primary defect (reduction in number of ommatidia, reduction in size of ommatidia, or both). "S" stands for "small" (i.e. pinhead mutations), "B" indicates "big" (i.e. bighead mutations). Single arrow signifies moderate change, two arrows indicate severe change.

Figure 2 Phenotypes of pinhead mutations.

Scanning electron micrographs of mutant heads displaying a size reduction. Each complementation group is represented by an allele of average strength. (A) wild-type, (B) *Inr*, (C) *Akt*, (D) *Rheb*, (E) *MASK*, (F) S3A, (G) S3B, (H) *RhoGAP88C*. Anterior is to the left.

Figure 3 Phenotypes of bighead mutations.

Scanning electron micrographs of heads with increased size. One representative allele is shown for each complementation group. (A) wild-type, (B) *Hairless*, (C) *crumbs*, (D) *Tsc1*, (E) *salvador*, (F) *lats*/*warts*, (G) *CSK*, (H) *ELP*. Anterior is to the left. Note the almost complete loss of interommatidial bristles in (B).

LITERATURE CITED

AHMED, Z., and T. S. PILLAY, 2001 Functional effects of APS and SH2-B on insulin receptor signalling. Biochem Soc Trans **29:** 529-534.

AHMED, Z., B. J. SMITH, K. KOTANI, P. WILDEN and T. S. PILLAY, 1999 APS, an adapter protein with a PH and SH2 domain, is a substrate for the insulin receptor kinase. Biochem J **341:** 665-668.

AHMED, Z., B. J. SMITH and T. S. PILLAY, 2000 The APS adapter protein couples the insulin receptor to the phosphorylation of c-Cbl and facilitates ligand-stimulated ubiquitination of the insulin receptor. FEBS Lett **475:** 31-34.

BAKER, B. S., G. HOFF, T. C. KAUFMAN, M. F. WOLFNER and T. HAZELRIGG, 1991 The doublesex locus of Drosophila melanogaster and its flanking regions: a cytogenetic analysis. Genetics **127:** 125-138.

BANG, A. G., V. HARTENSTEIN and J. W. POSAKONY, 1991 Hairless is required for the development of adult sensory organ precursor cells in Drosophila. Development **111:** 89-104.

BANG, A. G., and J. W. POSAKONY, 1992 The Drosophila gene Hairless encodes a novel basic protein that controls alternative cell fates in adult sensory organ development. Genes Dev **6:** 1752-1769.

BHAT, M. A., S. IZADDOOST, Y. LU, K. O. CHO, K. W. CHOI *et al.*, 1999 Discs Lost, a novel multi-PDZ domain protein, establishes and maintains epithelial polarity. Cell **96:** 833-845.

BOHNI, R., J. RIESGO-ESCOVAR, S. OLDHAM, W. BROGIOLO, H. STOCKER *et al.*, 1999 Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. Cell **97:** 865-875.

BROGIOLO, W., H. STOCKER, T. IKEYA, F. RINTELEN, R. FERNANDEZ *et al.*, 2001 An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. Curr Biol **11:** 213-221.

BROU, C., F. LOGEAT, M. LECOURTOIS, J. VANDEKERCKHOVE, P. KOURILSKY *et al.*, 1994 Inhibition of the DNA-binding activity of Drosophila suppressor of hairless and of its human homolog, KBF2/RBP-J kappa, by direct protein- protein interaction with Drosophila hairless. Genes Dev **8:** 2491-2503.

CEMAN, S., V. BROWN and S. T. WARREN, 1999 Isolation of an FMRP-associated messenger ribonucleoprotein particle and identification of nucleolin and the fragile X-related proteins as components of the complex. Mol Cell Biol **19:** 7925-7932.

CHAO, J. L., Y. C. TSAI, S. J. CHIU and Y. H. SUN, 2004 Localized Notch signal acts through eyg and upd to promote global growth in Drosophila eye. Development **131:** 3839-3847.

CHARROUX, B., C. ANGELATS, L. FASANO, S. KERRIDGE and C. VOLA, 1999 The levels of the bancal product, a Drosophila homologue of vertebrate hnRNP K protein, affect cell proliferation and apoptosis in imaginal disc cells. Mol Cell Biol **19:** 7846-7856.

CHEADLE, J. P., M. P. REEVE, J. R. SAMPSON and D. J. KWIATKOWSKI, 2000 Molecular genetic advances in tuberous sclerosis. Hum Genet **107:** 97-114.

CHEN, C., J. JACK and R. S. GAROFALO, 1996 The Drosophila insulin receptor is required for normal growth. Endocrinology **137:** 846-856.

CHO, K. O., and K. W. CHOI, 1998 Fringe is essential for mirror symmetry and morphogenesis in the Drosophila eye. Nature **396:** 272-276.

DE CELIS, J. F., D. M. TYLER, J. DE CELIS and S. J. BRAY, 1998 Notch signalling mediates segmentation of the Drosophila leg. Development **125:** 4617-4626.

DEAK, P., M. M. OMAR, R. D. SAUNDERS, M. PAL, O. KOMONYI *et al.*, 1997 P-element insertion alleles of essential genes on the third chromosome of Drosophila melanogaster: correlation of physical and cytogenetic maps in chromosomal region 86E-87F. Genetics **147:** 1697-1722.

DESHLER, J. O., M. I. HIGHETT, T. ABRAMSON and B. J. SCHNAPP, 1998 A highly conserved RNA-binding protein for cytoplasmic mRNA localization in vertebrates. Curr Biol **8:** 489-496.

DOMINGUEZ, M., and J. F. DE CELIS, 1998 A dorsal/ventral boundary established by Notch controls growth and polarity in the Drosophila eye. Nature **396:** 276-278.

FENG, Y., D. ABSHER, D. E. EBERHART, V. BROWN, H. E. MALTER *et al.*, 1997a FMRP associates with polyribosomes as an mRNP, and the I304N mutation of severe fragile X syndrome abolishes this association. Mol Cell **1:** 109-118.

FENG, Y., C. A. GUTEKUNST, D. E. EBERHART, H. YI, S. T. WARREN *et al.*, 1997b Fragile X mental retardation protein: nucleocytoplasmic shuttling and association with somatodendritic ribosomes. J Neurosci **17:** 1539-1547.

FERNANDEZ, R., D. TABARINI, N. AZPIAZU, M. FRASCH and J. SCHLESSINGER, 1995 The Drosophila insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential. EMBO J **14:** 3373-3384.

GAO, X., T. P. NEUFELD and D. PAN, 2000 Drosophila PTEN regulates cell growth and proliferation through PI3K- dependent and -independent pathways. Dev Biol **221:** 404-418.

GAO, X., and D. PAN, 2001 TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. Genes Dev **15:** 1383-1392.

GAO, X., Y. ZHANG, P. ARRAZOLA, O. HINO, T. KOBAYASHI *et al.*, 2002 Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. Nat Cell Biol **4:** 699-704.

GARAMI, A., F. J. ZWARTKRUIS, T. NOBUKUNI, M. JOAQUIN, M. ROCCIO *et al.*, 2003 Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell **11:** 1457-1466.

GAROFALO, R. S., 2002 Genetic analysis of insulin signaling in Drosophila. Trends in Endocrinology & Metabolism **13:** 156-162.

GOBERDHAN, D. C., and C. WILSON, 2003 The functions of insulin signaling: size isn't everything, even in Drosophila. Differentiation **71:** 375-397.

GOBERDHAN, D. C. I., N. PARICIO, E. C. GOODMAN, M. MLODZIK and C. WILSON, 1999 Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. Genes Dev **13:** 3244-3258.

GRAWE, F., A. WODARZ, B. LEE, E. KNUST and H. SKAER, 1996 The Drosophila genes crumbs and stardust are involved in the biogenesis of adherens junctions. Development **122:** 951-959.

HALFAR, K., C. ROMMEL, H. STOCKER and E. HAFEN, 2001 Ras controls growth, survival and differentiation in the Drosophila eye by different thresholds of MAP kinase activity. Development **128:** 1687-1696.

HARVEY, K. F., C. M. PFLEGER and I. K. HARIHARAN, 2003 The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell **114:** 457-467.

HAVIN, L., A. GIT, Z. ELISHA, F. OBERMAN, K. YANIV *et al.*, 1998 RNA-binding protein conserved in both microtubule- and microfilament- based RNA localization. Genes Dev **12:** 1593-1598.

HORI, T., A. TAKAORI-KONDO, Y. KAMIKUBO and T. UCHIYAMA, 2000 Molecular cloning of a novel human protein kinase, kpm, that is homologous to warts/lats, a Drosophila tumor suppressor. Oncogene **19:** 3101-3109.

HU, J., J. LIU, R. GHIRLANDO, A. R. SALTIEL and S. R. HUBBARD, 2003 Structural basis for recruitment of the adaptor protein APS to the activated insulin receptor. Mol Cell **12:** 1379-1389.

HUANG, H., C. J. POTTER, W. TAO, D. M. LI, W. BROGIOLO *et al.*, 1999 PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development. Development **126:** 5365-5372.

HUANG, X., Y. LI, K. TANAKA, K. G. MOORE and J. I. HAYASHI, 1995 Cloning and characterization of Lnk, a signal transduction protein that links T-cell receptor activation signal to phospholipase C gamma 1, Grb2, and phosphatidylinositol 3-kinase. Proc Natl Acad Sci U S A **92:** 11618-11622.

INOKI, K., Y. LI, T. XU and K. L. GUAN, 2003 Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev **17:** 1829-1834.

ITO, N., and G. M. RUBIN, 1999 gigas, a Drosophila homolog of tuberous sclerosis gene product-2, regulates the cell cycle. Cell **96:** 529-539.

JUSTICE, R. W., O. ZILIAN, D. F. WOODS, M. NOLL and P. J. BRYANT, 1995 The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes & Development **9:** 534-546.

KANGO-SINGH, M., R. NOLO, C. TAO, P. VERSTREKEN, P. R. HIESINGER *et al.*, 2002 Shar-pei mediates cell proliferation arrest during imaginal disc growth in Drosophila. Development **129:** 5719-5730.

KENYON, K. L., S. S. RANADE, J. CURTISS, M. MLODZIK and F. PIGNONI, 2003 Coordinating proliferation and tissue specification to promote regional identity in the Drosophila head. Dev Cell **5:** 403-414.

KOTANI, K., P. WILDEN and T. S. PILLAY, 1998 SH2-Balpha is an insulin-receptor adapter protein and substrate that interacts with the activation loop of the insulin-receptor kinase. Biochem J **335 (Pt 1):** 103-109.

LASKO, P., 2000 The Drosophila melanogaster genome: translation factors and RNA binding proteins. J Cell Biol **150:** F51-56.

MAIER, D., G. STUMM, K. KUHN and A. PREISS, 1992 Hairless, a Drosophila gene involved in neural development, encodes a novel, serine rich protein. Mech Dev **38:** 143-156.

MCPHERSON, J. P., L. TAMBLYN, A. ELIA, E. MIGON, A. SHEHABELDIN *et al.*, 2004 Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity. Embo J **23:** 3677-3688.

MINAMI, A., M. ISEKI, K. KISHI, M. WANG, M. OGURA *et al.*, 2003 Increased insulin sensitivity and hypoinsulinemia in APS knockout mice. Diabetes **52:** 2657-2665.

MONTAGNE, J., M. J. STEWART, H. STOCKER, E. HAFEN, S. C. KOZMA *et al.*, 1999 Drosophila S6 kinase: a regulator of cell size. Science **285:** 2126-2129.

NABEL-ROSEN, H., N. DOREVITCH, A. REUVENY and T. VOLK, 1999 The balance between two isoforms of the Drosophila RNA-binding protein how controls tendon cell differentiation. Mol Cell **4:** 573-584.

NAIRZ, K., H. STOCKER, B. SCHINDELHOLZ and E. HAFEN, 2002 High-resolution SNP mapping by denaturing HPLC. Proc Natl Acad Sci U S A **99:** 10575-10580.

NELLIST, M., M. A. VAN SLEGTENHORST, M. GOEDBLOED, A. M. VAN DEN OUWELAND, D. J. HALLEY *et al.*, 1999 Characterization of the cytosolic tuberin-hamartin complex. Tuberin is a cytosolic chaperone for hamartin. J Biol Chem **274:** 35647-35652.

NEUFELD, T. P., A. F. DE LA CRUZ, L. A. JOHNSTON and B. A. EDGAR, 1998 Coordination of growth and cell division in the Drosophila wing. Cell **93:** 1183-1193.

NEWSOME, T. P., B. ASLING and B. J. DICKSON, 2000 Analysis of Drosophila photoreceptor axon guidance in eye-specific mosaics. Development **127:** 851-860.

NURSE, P., 1975 Genetic control of cell size at cell division in yeast. Nature **256:** 547-551.

OHTSUKA, S., S. TAKAKI, M. ISEKI, K. MIYOSHI, N. NAKAGATA *et al.*, 2002 SH2-B is required for both male and female reproduction. Mol Cell Biol **22:** 3066-3077.

PANTALACCI, S., N. TAPON and P. LEOPOLD, 2003 The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nat Cell Biol **5:** 921-927.

PAPAYANNOPOULOS, V., A. TOMLINSON, V. M. PANIN, C. RAUSKOLB and K. D. IRVINE, 1998 Dorsal-ventral signaling in the Drosophila eye. Science **281:** 2031-2034.

PLANK, T. L., R. S. YEUNG and E. P. HENSKE, 1998 Hamartin, the product of the tuberous sclerosis 1 (TSC1) gene, interacts with tuberin and appears to be localized to cytoplasmic vesicles. Cancer Res **58:** 4766-4770.

POTTER, C. J., H. HUANG and T. XU, 2001 Drosophila tsc1 functions with tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. Cell **105:** 357-368.

RADIMERSKI, T., J. MONTAGNE, M. HEMMINGS-MIESZCZAK and G. THOMAS, 2002 Lethality of Drosophila lacking TSC tumor suppressor function rescued by reducing dS6K signaling. Genes Dev **16:** 2627-2632.

RAUSKOLB, C., and K. D. IRVINE, 1999 Notch-mediated segmentation and growth control of the Drosophila leg. Developmental Biology (Orlando) **210:** 339-350.

ROSS, A. F., Y. OLEYNIKOV, E. H. KISLAUSKIS, K. L. TANEJA and R. H. SINGER, 1997 Characterization of a beta-actin mRNA zipcode-binding protein. Mol Cell Biol **17:** 2158-2165.

SCANGA, S. E., L. RUEL, R. C. BINARI, B. SNOW, V. STAMBOLIC *et al.*, 2000 The conserved PI3'K/PTEN/Akt signaling pathway regulates both cell size and survival in Drosophila. Oncogene **19:** 3971-3977.

SCHWEISGUTH, F., and M. LECOURTOIS, 1998 The activity of Drosophila Hairless is required in pupae but not in embryos to inhibit Notch signal transduction. Dev Genes Evol **208:** 19-27.

SEDGWICK, S. G., and S. J. SMERDON, 1999 The ankyrin repeat: a diversity of interactions on a common structural framework. Trends Biochem Sci **24:** 311-316.

SIOMI, H., M. CHOI, M. C. SIOMI, R. L. NUSSBAUM and G. DREYFUSS, 1994 Essential role for KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. Cell **77:** 33-39.

SMITH, R. K., P. M. CARROLL, J. D. ALLARD and M. A. SIMON, 2002 MASK, a large ankyrin repeat and KH domain-containing protein involved in Drosophila receptor tyrosine kinase signaling. Development **129:** 71-82.

ST JOHN, M. A., W. TAO, X. FEI, R. FUKUMOTO, M. L. CARCANGIU *et al.*, 1999 Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. Nat Genet **21:** 182-186.

STAVELEY, B. E., L. RUEL, J. JIN, V. STAMBOLIC, F. G. MASTRONARDI *et al.*, 1998 Genetic analysis of protein kinase B (AKT) in Drosophila. Curr Biol **8:** 599-602.

STEWART, R. A., D. M. LI, H. HUANG and T. XU, 2003 A genetic screen for modifiers of the lats tumor suppressor gene identifies C-terminal Src kinase as a regulator of cell proliferation in Drosophila. Oncogene **22:** 6436-6444.

STOCKER, H., and E. HAFEN, 2000 Genetic control of cell size. Curr Opin Genet Dev **10:** 529-535.

STOCKER, H., T. RADIMERSKI, B. SCHINDELHOLZ, F. WITTWER, P. BELAWAT *et al.*, 2003 Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. Nat Cell Biol **5:** 559-565.

TAKAKI, S., K. SAUER, B. M. IRITANI, S. CHIEN, Y. EBIHARA *et al.*, 2000 Control of B cell production by the adaptor protein lnk. Definition Of a conserved family of signal-modulating proteins. Immunity **13:** 599-609.

TAO, W., S. ZHANG, G. S. TURENCHALK, R. A. STEWART, M. A. ST JOHN *et al.*, 1999 Human homologue of the Drosophila melanogaster lats tumour suppressor modulates CDC2 activity. Nat Genet **21:** 177-181.

TAPON, N., K. F. HARVEY, D. W. BELL, D. C. WAHRER, T. A. SCHIRIPO *et al.*, 2002 salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. Cell **110:** 467-478.

TAPON, N., N. ITO, B. J. DICKSON, J. E. TREISMAN and I. K. HARIHARAN, 2001 The drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. Cell **105:** 345-355.

TEE, A. R., B. D. MANNING, P. P. ROUX, L. C. CANTLEY and J. BLENIS, 2003 Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol **13:** 1259-1268.

TEPASS, U., 1996 Crumbs, a component of the apical membrane, is required for zonula adherens formation in primary epithelia of Drosophila. Dev Biol **177:** 217-225.

TEPASS, U., C. THERES and E. KNUST, 1990 crumbs encodes an EGF-like protein expressed on apical membranes of Drosophila epithelial cells and required for organization of epithelia. Cell **61:** 787-799.

UDAN, R. S., M. KANGO-SINGH, R. NOLO, C. TAO and G. HALDER, 2003 Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nat Cell Biol **5:** 914-920.

VAN SLEGTENHORST, M., R. DE HOOGT, C. HERMANS, M. NELLIST, B. JANSSEN *et al.*, 1997 Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. Science **277:** 805-808.

VAN SLEGTENHORST, M., M. NELLIST, B. NAGELKERKEN, J. CHEADLE, R. SNELL *et al.*, 1998 Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products. Hum Mol Genet **7:** 1053-1057.

VERDU, J., M. A. BURATOVICH, E. L. WILDER and M. J. BIRNBAUM, 1999 Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB. Nat Cell Biol **1:** 500-506.

WEIGMANN, K., S. M. COHEN and C. F. LEHNER, 1997 Cell cycle progression, growth and patterning in imaginal discs despite inhibition of cell division after inactivation of Drosophila Cdc2 kinase. Development **124:** 3555-3563.

WEINKOVE, D., and S. J. LEEVERS, 2000 The genetic control of organ growth: insights from Drosophila. Curr Opin Genet Dev **10:** 75-80.

WEINKOVE, D., T. P. NEUFELD, T. TWARDZIK, M. D. WATERFIELD and S. J. LEEVERS, 1999 Regulation of imaginal disc cell size, cell number and organ size by Drosophila class I(A) phosphoinositide 3-kinase and its adaptor. Curr Biol **9:** 1019-1029.

WODARZ, A., U. HINZ, M. ENGELBERT and E. KNUST, 1995 Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of Drosophila. Cell **82:** 67-76.

WU, S., J. HUANG, J. DONG and D. PAN, 2003 hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell **114:** 445-456.

XIA, H., H. QI, Y. LI, J. PEI, J. BARTON *et al.*, 2002 LATS1 tumor suppressor regulates G2/M transition and apoptosis. Oncogene **21:** 1233-1241.

XU, T., W. WANG, S. ZHANG, R. A. STEWART and W. YU, 1995 Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. Development **121:** 1053-1063.

YABUTA, N., T. FUJII, N. G. COPELAND, D. J. GILBERT, N. A. JENKINS *et al.*, 2000 Structure, expression, and chromosome mapping of LATS2, a mammalian homologue of the Drosophila tumor suppressor gene lats/warts. Genomics **63:** 263-270.

YANG, X., D. M. LI, W. CHEN and T. XU, 2001 Human homologue of Drosophila lats, LATS1, negatively regulate growth by inducing G(2)/M arrest or apoptosis. Oncogene **20:** 6516-6523.

YOKOUCHI, M., R. SUZUKI, M. MASUHARA, S. KOMIYA, A. INOUE *et al.*, 1997 Cloning and characterization of APS, an adaptor molecule containing PH and SH2 domains that is tyrosine phosphorylated upon B-cell receptor stimulation. Oncogene **15:** 7-15.

ZHANG, Y., X. GAO, L. J. SAUCEDO, B. RU, B. A. EDGAR *et al.*, 2003 Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol **5:** 578-581.

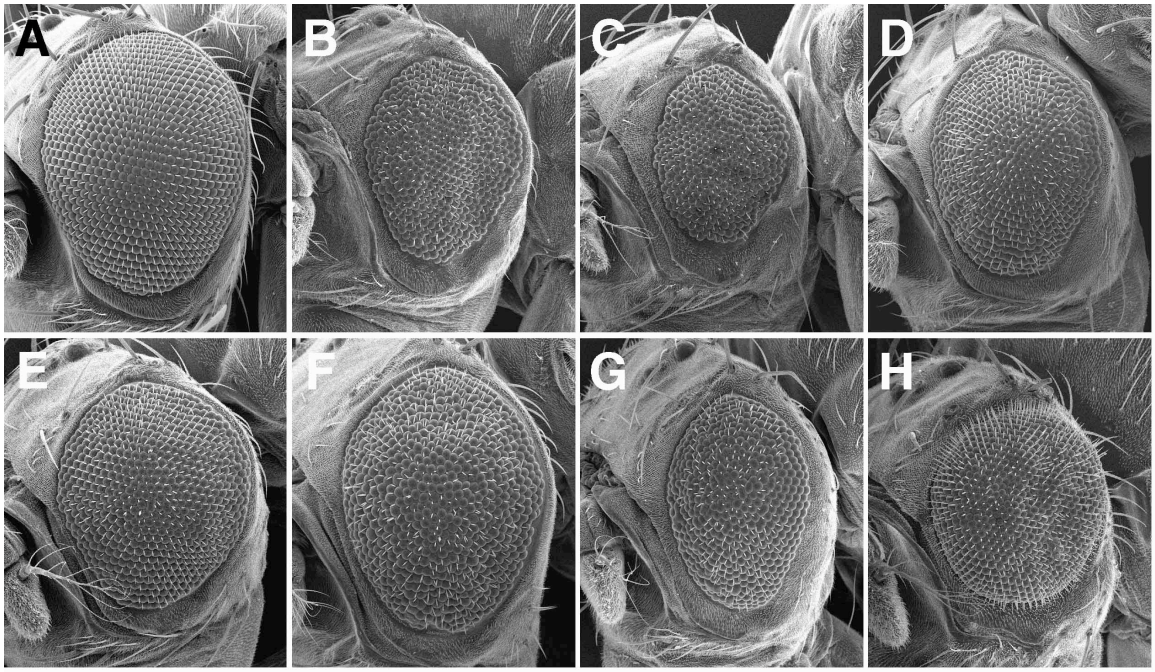

Figure 2

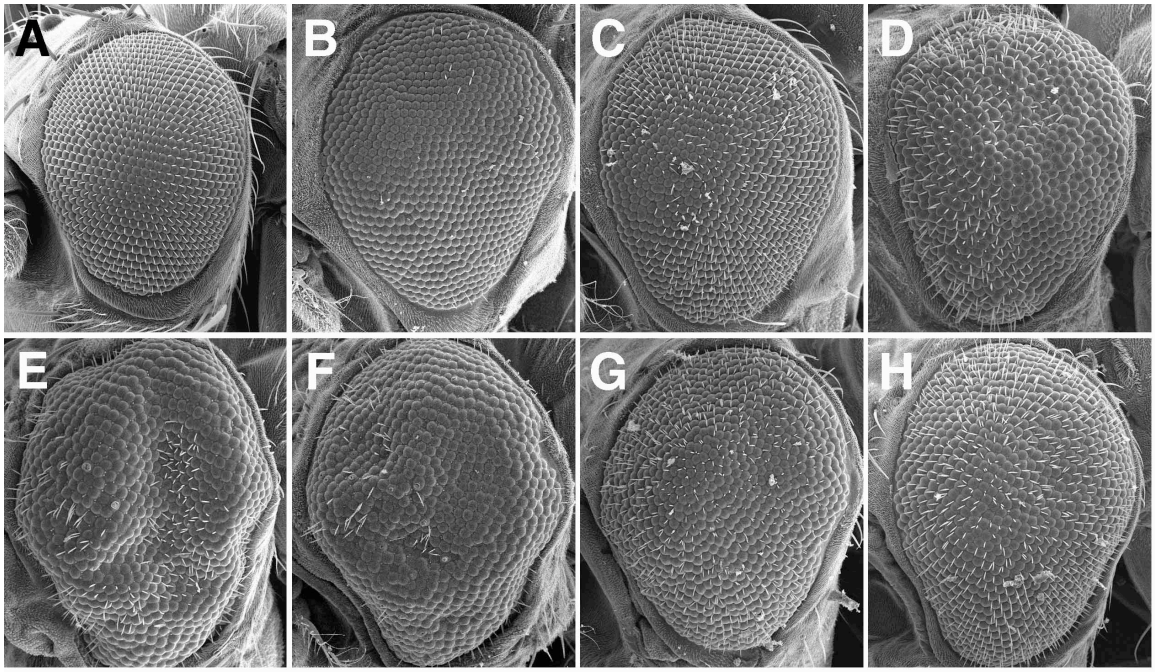

Figure 3

Rheb is an essential regulator of S6K in controlling cell growth in *Drosophila*

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Rheb is an essential regulator of S6K in controlling cell growth in *Drosophila*

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Understanding the mechanisms through which multicellular organisms regulate cell, organ and body growth is of relevance to developmental biology and to research on growth-related diseases such as cancer. Here we describe a new effector in growth control, the small GTPase Rheb (Ras homologue enriched in brain). Mutations in the *Drosophila melanogaster Rheb* gene were isolated as growth-inhibitors, whereas overexpression of *Rheb* promoted cell growth. Our genetic and biochemical analyses suggest that Rheb functions downstream of the tumour suppressors Tsc1 (tuberous sclerosis 1)/Tsc2 in the TOR (target of rapamycin) signalling pathway to control growth, and that a major effector of Rheb function is S6K.

A growing number of studies in genetically tractable model organisms, such as *D. melanogaster*, have greatly enhanced our understanding of growth regulation. From these efforts, two highly conserved signalling pathways dedicated to the control of growth have emerged, namely the insulin receptor (InR)/phosphatidylinositol-3-OH kinase (PI(3)K) and TOR pathways[1,2]. Recent studies have also shown that these two pathways interact, although the mechanisms by which they communicate are the subject of controversy[3,4]. In addition, each pathway seems to be modulated by distinct tumour suppressor genes, *PTEN* (phosphatase and tensin homologue deleted in chromosome 10) and *TSC1*/*TSC2*, respectively[5,6]. Whereas it is clear that PTEN constrains PI(3)K signalling by dephosphorylation of phosphatidylinositol-3,4,5-triphosphate ($PtdInsP_3$)[7], the mechanism by which TSC1 and TSC2 counteract TOR signalling remains elusive. Importantly, TSC2 possesses a putative GTPase-activating protein (GAP) domain, which has been shown to increase the intrinsic GTPase activity of the small GTPases Rap1 and Rab5 (refs 8, 9). Here, we present genetic and biochemical data from *Drosophila* suggesting a novel role for the small GTPase Rheb in the TOR/S6K signalling pathway.

To identify growth-regulating genes, we performed two complementary screens for loss- and gain-of-function mutations, respectively. In the loss-of-function screen, we discovered a novel complementation group of ten alleles that impair cell and organ growth. The ethylmethane sulphonate (EMS)-induced mutations were identified on the basis of reduced head size of mosaic animals consisting of heads largely made up of homozygous mutant cells and bodies containing heterozygous cells (compare Fig. 1a with Fig. 1b). This phenotype is reminiscent of mutations in InR signalling components. The heads of mutant flies were largely made up of homozygous mutant cells and bodies containing heterozygous cells (Fig. 1a), a phenotype reminiscent of mutations in InR signalling components[10,11]. Genetic mapping of two representative alleles and subsequent testing of candidate open reading frames identified alterations in the gene *CG1081* in seven alleles. *CG1081* encodes a small GTPase most closely related to mammalian Rheb[12] (Supplementary Information, Fig. S1a). Therefore, we named this complementation group *Rheb*.

The gain-of-function screen for genes that stimulate growth when overexpressed resulted in the identification of an EP element in the *Rheb* locus (EP 50.084). EP-mediated overexpression of *Rheb* in the developing eye substantially increased eye size (Fig. 1c). We generated six additional *Rheb* loss-of-function alleles by imprecise excision of EP 50.084. Whereas all combinations of the EMS-induced *Rheb* alleles were lethal, some hetero-allelic combinations of EMS-induced alleles and EP excision alleles were viable and resulted in flies of reduced size (Supplementary Information, Fig. S1b). The size reduction was caused by a decrease in cell number (3–11%), as well as in cell size (9–14% in wing cells, more than 20% in eye cells as judged by ommatidial size). In addition, the small flies eclosed with a delay of at least one day and the females had rudimentary ovaries and were sterile (Fig. 1d). Thus, the surviving *Rheb* mutant flies display all the hallmarks of impaired InR signalling activity, resembling flies lacking the insulin-receptor substrate (IRS) protein Chico[10].

A more severe reduction in Rheb function (in heteroallelic combinations of *Rheb* mutations) was lethal at late larval or early pupal stages. Mutant larvae and pupae were consistently smaller (Fig. 1e, f), although the phenotype was variable. Interestingly, the size reduction was more pronounced in the endoreplicative larval tissue than in the imaginal discs (Fig. 1g–j), similarly to the larval phenotype of *TOR* mutants[13,14]. Staining of DNA in salivary glands and fat body cells demonstrated a severe deficit in endoreplication (Fig. 1j).

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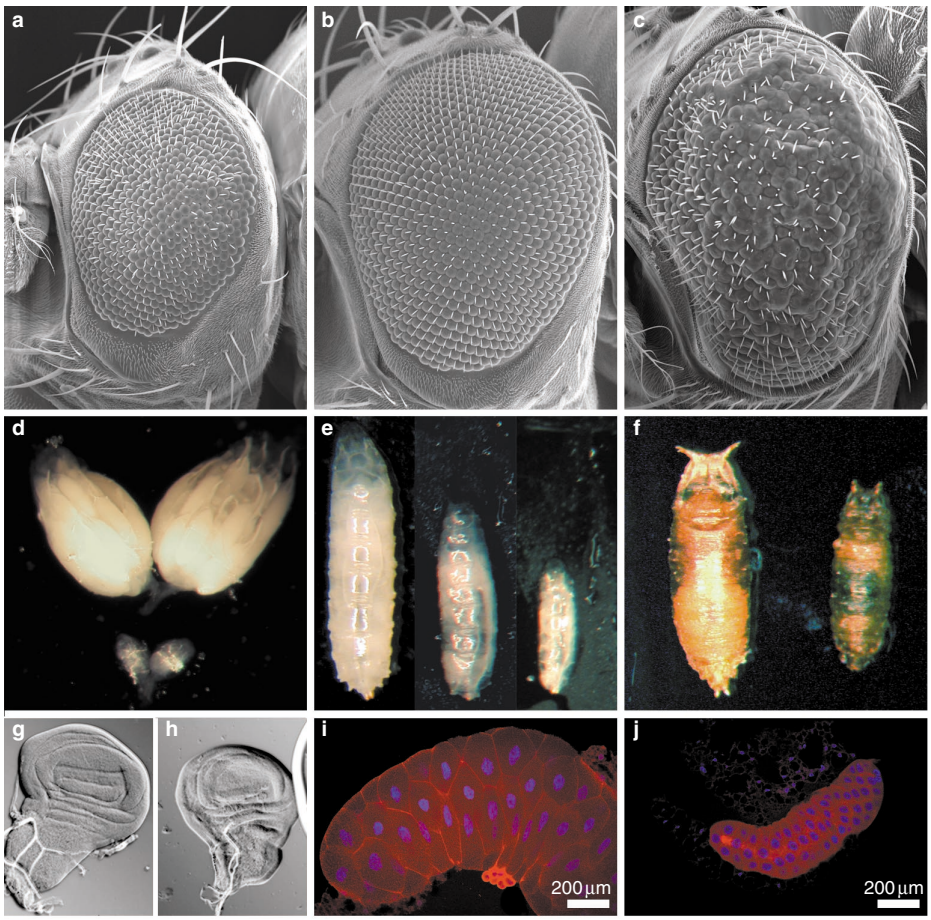

Figure 1 Growth defects caused by *Rheb* mutations. (**a**–**c**) Phenotypes that resulted in the identification of *Rheb* alleles. Shown are SEMs of female fly eyes taken at the same magnification. EMS-induced *Rheb* loss-of-function alleles were identified on the basis of the pinhead phenotype in mosaic animals with largely homozygous mutant heads, as shown in **a**. A comparison with a control eye, as shown in **b**, demonstrates the marked size reduction. An EP-element driving *Rheb* expression was isolated as a gain-of-function mutation promoting overgrowth in the eye, as shown in **c**. (**d**) Ovaries of viable $Rheb^{7A1}/Rheb^{44.1}$ females (bottom) are markedly reduced in size when compared with control ovaries (top). (**e**, **f**) Phenotype of a pupal lethal heteroallelic combination ($Rheb^{7A1}/Rheb^{26.2}$). The size range of mutant larvae (**e**, middle and right) is shown in comparison with a control larva (left). Whereas the smaller mutant larvae die, the larger mutant larvae arrest development at the early pupal stage. A corresponding pupa (**f**, right) is clearly smaller than the control pupa (left). (**g**–**j**) Size defects of imaginal discs and salivary glands in larvae of a pupal lethal heteroallelic combination ($Rheb^{3M2}/Rheb^{26.2}$). Whereas the size reduction of mutant imaginal discs is roughly proportional to the larval size (**g**, control; **h**, mutant), the salivary glands are more severely reduced (compare **j** to control gland in **i**). DAPI staining (blue) shows a strong endoreplication deficit in *Rheb* mutant salivary glands. Membranes are stained with an anti-lin7 antibody (red).

The behaviour of *Rheb* mutant cells was studied during development by means of mitotic recombination. Clones of cells homozygous for EMS-induced *Rheb* alleles grew poorly and were consistently smaller than their corresponding sister clones (Fig. 2a). When provided with a proliferative advantage (by means of the Minute technique), *Rheb* mutant cells still failed to cover large regions of the imaginal discs. Instead, the resulting clones typically displayed elongated shapes with thin extensions (Supplementary Information, Fig. S2a). A possible explanation for this unusual phenotype may reside in their attempt to minimise contact with other mutant cells. To our knowledge, this phenomenon has not been previously described in the context of growth-regulating genes. Despite this abnormal behaviour, *Rheb* mutant cells differentiate properly into adult structures. For example, analysis of clones in the adult eye revealed the presence of extremely small photoreceptor cells of otherwise normal structure and arrangement in the mutant tissue (Fig. 2b). The size reduction phenotype is strictly cell-autonomous. Taken together, the characterisation of the mutant phenotypes demonstrates that *Rheb* is required for proper growth regulation in a cell-autonomous manner.

Next, we examined whether overexpression of *Rheb* is sufficient to promote growth. The effect of overexpressing *Rheb* during development through the use of the EP 50.084 line and two independent UAS-*Rheb* lines was monitored in marked clones in imaginal discs and in the adult eye. All the lines yielded qualitatively similar results, with the EP line consistently showing the strongest effects. Clones overexpressing *Rheb* in the wing imaginal disc attained a substantially larger size when compared with control clones (Fig. 2c). This enlargement is caused by a significant increase in cell size (a 48% increase in area covered per cell). In contrast, the cell doubling time remained unchanged in cells expressing *Rheb* versus control cells (10.5 h versus 10.8 h). Consistent with the size effect in the imaginal discs, cells expressing *Rheb* in differentiating cells posterior to the morphogenetic furrow (under the control of GMR regulatory sequences) resulted in enlarged but fully differentiated photoreceptor cells (a 66% size increase of the

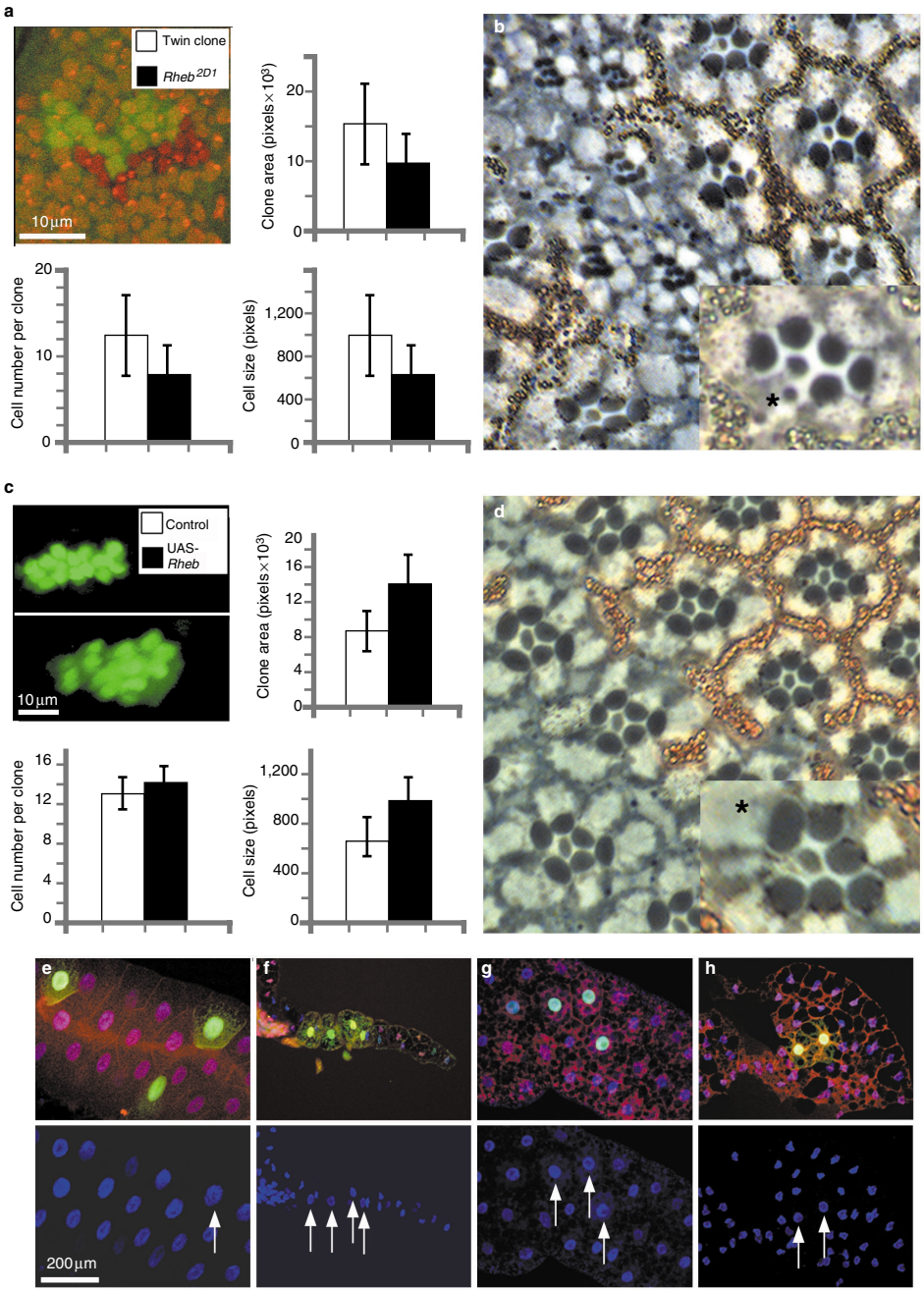

Figure 2 Clonal analysis of Rheb function. (**a**) Loss-of-function clones in the wing imaginal disc are consistently smaller than their corresponding twin clones. A clone of cells homozygous mutant for *Rheb*^{2D1} (marked by the absence of GFP) is only about half the size of its twin spot (bright green staining). Nuclei are labelled by DAPI staining (shown in red). The reduced clone size is accounted for by fewer and smaller cells ($n = 10$, $P = 0.00038$ for cell number, $P = 0.00126$ for cell size). (**b**) Tangential sections through compound eyes reveal that cells homozygous mutant for *Rheb*^{3M2} (marked by the lack of pigments) are able to differentiate into the various cell types of the eye. The size of the mutant cells, however, is greatly reduced (as reflected by a 72% reduction in rhabdomere area in tangential sections). A mosaic ommatidium containing a single mutant photoreceptor cell (inset, asterisk) demonstrates the cell-autonomy of the size reduction. (**c**) Flp-out clones of *Rheb*-expressing cells in the wing imaginal disc attain a significantly larger size. Clones 40 h after induction and marked by GFP expression are shown (top: control, bottom: expressing *Rheb*). The increase in clonal area is caused by larger cells ($n = 20$, $P = 0.00002$), whereas the number of cells is not significantly altered ($P = 0.087$). (**d**) Expression of *Rheb* during eye development results in an enlargement of photoreceptor cells.Cells expressing *Rheb* under GMR-control are marked by the absence of pigmentation. Cell-autonomy of the size increase is demonstrated by a single *Rheb*-expressing photoreceptor cell (asterisk) in a mosaic ommatidium (inset). (**e**–**h**) The effects of *Rheb* expression in endoreplicative larval tissues under normal conditions (**e**, **g**) and under amino-acid deprivation (**f**, **h**). Cells expressing *Rheb* (labelled with GFP, arrows in lower panels) under normal nutrient availability in salivary glands (**e**) and fat body cells (**g**) display only a mild size increase. In larvae starved for amino acids, however, *Rheb* expression exerts strong effects on both cell size and DNA ploidy. In salivary glands (**f**), *Rheb*-expressing cells display a several fold increase in size and contain much more DNA (stained with DAPI, lower panels) than non-expressing neighbouring cells, but they do not reach normal size and ploidy. In the fat body cells (**h**), expression of *Rheb* reverts the starvation phenotype completely. Size, DNA content, and appearance (amount of vesicles) of these cells are indistinguishable from non-starved cells. Membranes are visualised with an anti-lin7 antibody staining (red). Separate DAPI staining is shown in lower panels (blue).

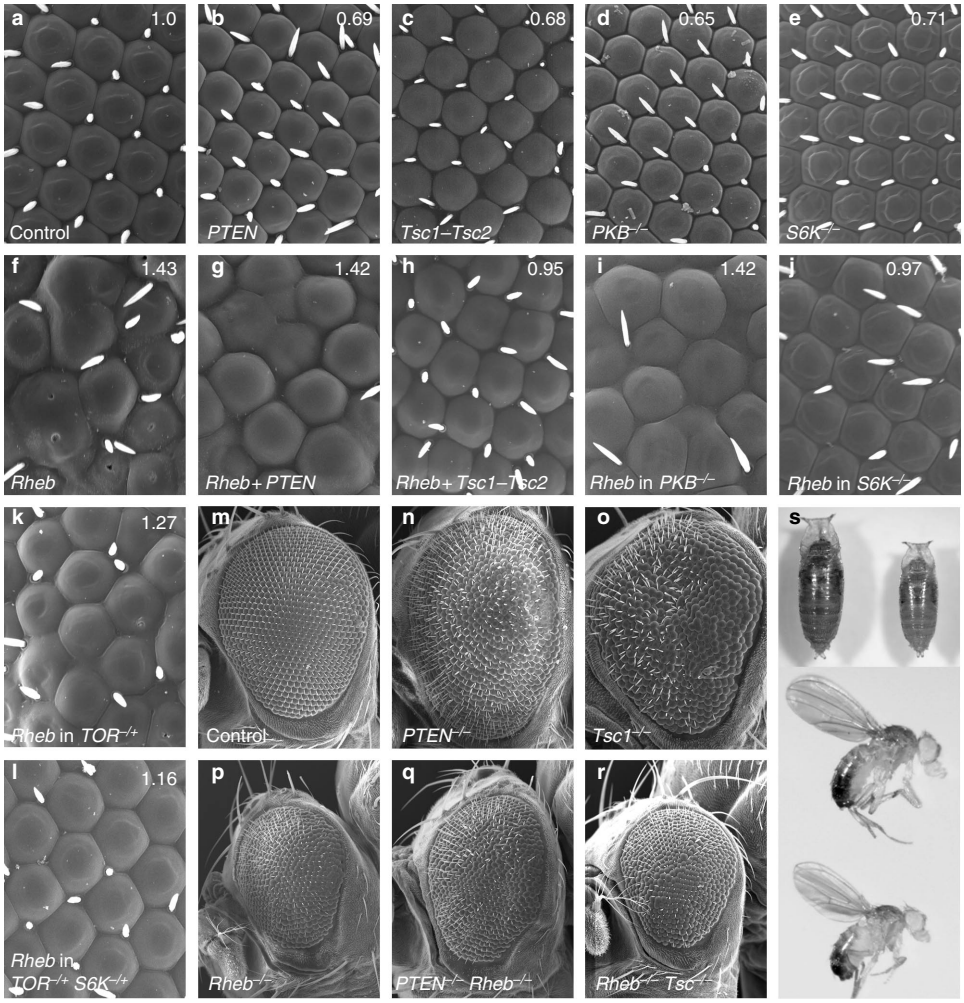

Figure 3 Epistatic relationship of Rheb with the InR/dPI(3)K and the TOR signalling pathways. (**a–l**) SEMs showing ommatidial size and shape of female flies. (**a**) Control (**b**) GMR-*Gal4*/+; UAS-*PTEN*/+ (**c**) GMR-*Gal4*/+; UAS-*Tsc1* UAS-*Tsc2*/+ (**d**) $dPKB^{1}/dPKB^{3}$ (**e**) $S6K^{l\text{-}1}/S6K^{l\text{-}1}$ (**f**) GMR-*Gal4*/+; EP50.084/+ (**g**) GMR-*Gal4*/+; EP50.084/UAS-*PTEN* (**h**) GMR-*Gal4*/+; EP50.084/UAS-*Tsc1* UAS-*Tsc2* (**i**) GMR-*Gal4*/+; EP50.084 $dPKB^{1}/dPKB^{3}$ (**j**) GMR-*Gal4*/+; $S6K^{l\text{-}1}$ EP50.084/$S6K^{l\text{-}1}$ (**k**) GMR-*Gal4*/TOR^{2L1}; EP50.084/+ (**l**) GMR-*Gal4*/TOR^{2L1}; EP50.084/$S6K^{l\text{-}1}$. Ommatidial size relative to the control is indicated in each panel. Expression of *Rheb* under GMR-*Gal4* control results in large and disorganised ommatidia (**f**). This phenotype can be suppressed by co-expression of *Tsc1/2* (**h**), but not by *PTEN* (**g**). The enlarged ommatidial size is still evident in a *dPKB* mutant background (**i**), but is neutralised in flies lacking S6K function (**j**). The effects of *Rheb* overexpression are dominantly alleviated by TOR^{2L1} (**k**). (**m–r**) SEMs showing heads of mosaic female flies generated by the ey-Flp method. The heads are largely homozygous mutant for (**m**) Control (**n**) $PTEN^{2L117}$ (**o**) $Tsc1^{2G3}$ (**p**) $Rheb^{2D1}$ (**q**) $PTEN^{2L117}$ and $Rheb^{2D1}$ (**r**) $Rheb^{2D1}$ and $Tsc1^{2G3}$. Note that *Rheb* is epistatic over both *PTEN* and *Tsc1*, as indicated by the Rheb-like phenotype of the respective double mutants as shown in **q** and **r**. (**s**) Pupae doubly mutant for *Tsc1* and *Rheb* (upper panel, right) are smaller than heterozygous control pupae (left). Animals lacking Tsc1 do survive if Rheb function is compromised (lower panel, bottom). The surviving flies are slightly reduced in size when compared with the heterozygous control (top). Genotypes are: $Tsc1^{2X1}$, $Rheb^{2D1}/TM3$ (heterozygous control animals), $Tsc1^{2X1}$, $Rheb^{2D1}/Tsc1^{1A2}$, $Rheb^{7A1}$ (doubly mutant animals).

rhabdomeres; Fig. 2d). As in the case of the loss-of-function clones, the size alteration was cell-autonomous. Thus, *Rheb* is sufficient to promote cellular growth.

As both InR and TOR signalling have been implicated in the response to nutrient availability[15,16], we asked whether overexpression of *Rheb* would promote growth even under starvation conditions. It has been shown that depriving larvae of amino acids blocks endoreplication of the larval tissues, but that this can be overcome by expression of Dp110/PI(3)K[16]. *Rheb* was expressed in small clones of cells in the salivary glands and in the fat body. Under normal food conditions, only a very subtle increase in cell size was observed (Fig. 2e, g). In larvae starved of amino acids, however, *Rheb* expression had a pronounced effect on both DNA content (as visualised by DAPI staining) and cell size (Fig. 2f, h). Despite the lack of amino acids, larval cells expressing *Rheb* reached a normal size in the fat body, and the size and endoreplication deficits were significantly alleviated in the salivary glands. We conclude that *Rheb* is sufficient to counteract the effects of amino-acid deprivation and thus may function in amino-acid sensing.

Given the similarities between *Rheb* and mutants in the InR and TOR signalling pathways, it is conceivable that Rheb represents a novel component of one of these growth control pathways. To test this possibility, a detailed epistasis analysis was performed. First, we tested whether the negative regulators of InR and TOR signalling — PTEN and Tsc1–Tsc2, respectively — could counteract the effects of *Rheb*

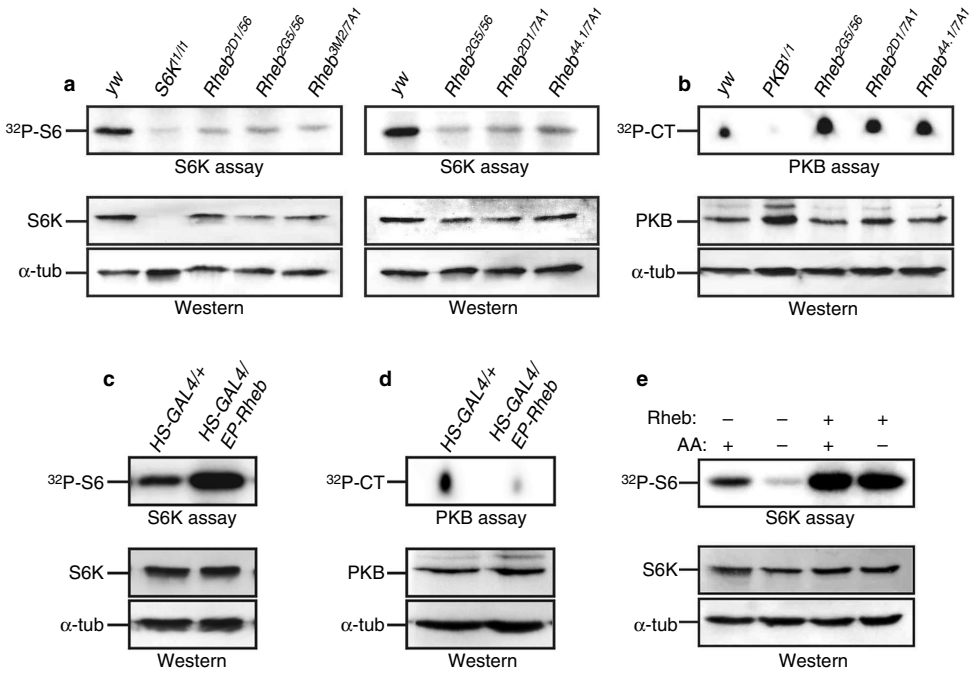

Figure 4 Rheb affects the kinase activities of S6K and dPKB. (**a**) S6K activity depends on Rheb function. Larval extracts of various *Rheb* heteroallelic combinations were assayed for S6K kinase activity. All combinations showed a significant reduction in the kinase activity. (**b**) In contrast to the effect on S6K, reduction of Rheb function resulted in an increase in dPKB activity. (**c**) Ubiquitous expression of *Rheb* resulted in a marked increase in S6K activity, accompanied by a decrease in dPKB activity (**d**). Kinase assays were performed 7 h after heat-shock-mediated *Rheb* induction. (**e**) Amino-acid deprivation for 6 h (after 4 h recovery from heat-shock) did not compromise the ability of Rheb to stimulate S6K activity. Western blots of S6K and α-tubulin are shown as loading controls in the bottom panels of **a**, **c** and **e**. ^{32}P-CT: ^{32}P-Crosstide. Western blots of dPKB and α-tubulin are shown as loading controls in the bottom panels of **b** and **d**.

overexpression. All overexpression experiments were performed in the eye using the GMR-*Gal4* driver line. Expression of either *PTEN* or *Tsc1–Tsc2* alone resulted in a very similar size reduction of the ommatidia (Fig. 3b, c) when compared with control ommatidia (Fig. 3a)[17–20]. However, whereas expression of *PTEN* had no influence on the increase in ommatidial size caused by *Rheb* overexpression (Fig. 3g), co-expression of *Tsc1–Tsc2* resulted in ommatidia of approximately wild-type size (Fig. 3h), indicating that the activities of *Rheb* and *Tsc1–Tsc2* can counteract each other. Next, the enlarged ommatidia phenotype of GMR-*Rheb* was assayed in a number of mutant backgrounds. Reducing the activity of *Drosophila* protein kinase B (dPKB) had no effect on the ommatidial size (Fig. 3i). Similar results were obtained with hypomorphic mutations in *InR* and *Dp110*, respectively (data not shown). In contrast, ommatidial size was dominantly reduced by a mutation in *TOR* (*TOR*^{2L1}; Fig. 3k), and a suppression to wild-type size was observed in a *S6K* mutant background (Fig. 3j). Thus, the *Rheb* overexpression phenotype is dependent on TOR and S6K function, but is independent of InR signal strength. Finally, we analysed the behaviour of *Rheb PTEN* and *Rheb Tsc1* double mutants. The phenotypic consequences were assayed in mosaic animals using the ey-Flp method. As expected, the *Rheb PTEN* double-mutant tissue clearly displayed a *Rheb* phenotype (Fig. 3q). The *Rheb Tsc1* mutant tissue also resembled *Rheb* single mutants (Fig. 3r), indicating that *Rheb* is epistatic over *Tsc1*.

Complete loss of Tsc1 function results in larval lethality[17,18,21]. Importantly, we found that a simultaneous reduction of *Rheb* function was sufficient to restore viability. The emerging double-mutant flies displayed a weak *Rheb* hypomorphic phenotype (a moderate size reduction; Fig. 3s). These findings suggest that the major consequence of a lack of Tsc1 is overactivation of Rheb.

Our genetic analysis indicated that Rheb regulates S6K through TOR. Therefore, we tested whether S6K activity is dependent on Rheb function. Larval extracts of various heteroallelic *Rheb* combinations were subjected to S6K and PKB kinase assays. Indeed, S6K activity was significantly reduced in all combinations without any apparent effect on S6K protein levels (Fig. 4a). PKB activity, however, was consistently increased (Fig. 4b). This is in agreement with the hypothesis that S6K is an essential component of a negative feedback loop regulating InR signalling[22,23]. Conversely, ubiquitous expression of *Rheb* resulted in an increase in S6K activity (Fig. 4c) and a concomitant decrease in dPKB activity (Fig. 4d). The stimulation of S6K activity by Rheb was also observed after amino-acid deprivation (Fig. 4e). Thus, Rheb is both necessary and sufficient for S6K activation.

Although Rheb is essential for S6K activity, and the overgrowth phenotype elicited by *Rheb* overexpression depends on S6K, regulation of S6K is clearly not the only effect of Rheb activity. Whereas flies lacking S6K function are semi-viable (exhibiting a severe delay in development and a reduced body size[24]), loss of Rheb is lethal. Moreover, reduction of Rheb activity results in a decrease in cell number and cell size (as opposed to *S6K* mutants, which only affect cell size). Finally, the characteristic shape of *Rheb* mutant cell clones suggests that *Rheb* has other function in addition to growth control.

Two models of Rheb activity can be envisaged: first, Rheb could function in the TOR signalling pathway directly downstream of and negatively regulated by Tsc1–Tsc2 (Supplementary Information, Fig. S3a). Second, Rheb might be a component of an independent

pathway that impinges on S6K (Supplementary Information, Fig. S3b). In the latter model, the TOR signalling pathway and the putative parallel pathway would both be necessary for the full activation of S6K. This could explain why impairing the activity of one pathway interferes with the consequences of overactivating the other. Nevertheless, we favour the former model because *Rheb* mutants show striking similarities with TOR signalling defects and because of the intimate genetic interactions of *Rheb* with *Tsc1–Tsc2*. A particularly attractive hypothesis implicates Tsc2 as the GAP of Rheb. Indeed, in an accompanying paper, Zhang *et al.* provide evidence that Tsc2 is the GAP for Rheb in *Drosophila* (this issue), and the same conclusion has been derived from studies on the mammalian homologues of *Drosophila* TSC2 and Rheb (F.J.T. Zwartkruis, J.L. Bos and G.T., personal communication).

Interestingly, loss of *rhb1* function in the fission yeast *Schizosaccharomyces pombe* results in a growth arrest phenotype that is very similar to that of nitrogen-starved cells[25]. Thus, the function of Rheb in growth regulation in response to nutrients (amino acids) may have been conserved during evolution. Furthermore, the fact that impaired Rheb function is sufficient to suppress the phenotypic consequences of loss of PTEN and TSC1/TSC2 suggests that Rheb might be a suitable target for therapeutic intervention in a wide range of tumours. □

METHODS

Genetics. EMS-induced *Rheb* alleles were isolated in an *ey-Flp* mosaic screen[26] that will be described elsewhere. Two alleles, $Rheb^{2D1}$ and $Rheb^{2G5}$, were mapped genetically with respect to visible markers. A subsequent fine mapping step placed the mutations between the P-element insertions EP974 (83A3) and l(3)j1C2 (83C1-2) in a candidate region of 312.5 kb. Eight polymorphisms distributed over the candidate region were used to establish a high-resolution SNP map. The candidate region could be narrowed down to 60 kb by mapping the recombination sites between the *Rheb* alleles and the two P elements using SNP detection by DHPLC[27]. Candidate open reading frames in this interval were amplified by PCR from heterozygous *Rheb* flies and tested for polymorphisms by DHPLC. Amplified fragments of *CG1081* displayed various polymorphisms and were subsequently sequenced.

The EP line 50.084 was identified among 10,000 novel insertions of a double-headed EP element. It is inserted six nucleotides downstream of the 5′ end of the first exon of transcript CG1081-RA (GadFly database). The EP50.084 chromosome carries a closely linked lethal factor that cannot be reverted by mobilisation of the EP element. The UAS sites at the 5′ end of EP50.084 were excised by *Cre-loxP*-mediated recombination to yield a single-headed EP element capable of driving *Rheb*. EP50.084 was remobilised by crossing in the transposase source Δ2-3 to generate imprecise excision alleles. At least 83 independent reversions of the dominant $yellow^{+}$ marker were recovered; six of them failed to complement the *Rheb* alleles. The two imprecise excision alleles that yielded viable combinations with some EMS-alleles ($Rheb^{44.1}$ and $Rheb^{56}$) retained partial sequences of the EP element (0.6 and 1.1 kb, respectively) without deleting any flanking genomic sequences.

The following transgenes and mutations were used for interaction studies:

GMR-*Gal4* (ref. 28), UAS-*PTEN* (ref. 20), UAS-*Tsc1*, UAS-*Tsc2* (ref. 17), $dPKB^{1}$ (ref. 29), $dPKB^{3}$ (ref. 30), TOR^{2L1} (ref. 13), $S6K^{l-1}$ (ref. 24), $PTEN^{2L117}$ (ref. 31), $Tsc1^{2G3}$, $Tsc1^{2X1}$ and $Tsc1^{1A2}$ (H.S. and E.H., unpublished observations).

To generate loss-of-function clones, we used the lines *y w hs-Flp*; *FRT82 Ubi-GFP* and *y w hs-Flp*; *FRT82 Ubi-GFP M* / *TM6B* for clones in the imaginal discs, and *y w hs-Flp*; *FRT82* w^{+} *M* / *TM6B* for clones in the adult eye. Clones were induced in 48–72-h-old larvae by a 45-min heat-shock at 34 °C. To circumvent potential side effects caused by second hits, at least three different EMS-induced *Rheb* alleles were tested in each experiment. The results were always qualitatively similar.

Overexpression clones were generated by means of the 'FLP-out' technique using the lines *y w hs-Flp*; *Act>CD2>Gal4* UAS-*GFPnls* / *TM6B* (ref. 32) and *y w hs-Flp*; GMR>w^{+}>*Gal4* (ref. 33), respectively. FLP-out clones were induced either in 80-h-old larvae by a 13-min heat-shock at 34 °C (*Act>CD2>Gal4*), or in 24–48-h-old larvae by a 1-h heat-shock at 37 °C (GMR>w^{+}>*Gal4*). To achieve overexpression in the endoreplicative larval tissues, spontaneous FLP-out events using *y w hs-Flp*; *Act>CD2>Gal4* UAS-*GFPnls* / *TM6B* without heat-shock application were recovered[16]. All the overexpression experiments were performed using EP50.084 and two UAS-*Rheb* lines (individually or in combination). The severity of the resulting phenotypes was always in accordance with the order EP50.084 > UAS-*Rheb13.1+14.2* > UAS-*Rheb14.2* > UAS-*Rheb13.1*, presumably reflecting different expression levels. To deprive larvae of dietary amino acids, 60-h-old larvae were incubated in 20% sucrose solution.

Eye-specific clones were generated using the ey-Flp system, as previsouly described[26]. For the double-mutant analysis of *PTEN Rheb* clones, the ey-Flp systems for 2L and 3R were combined to induce clones simultaneously for *FRT40* $PTEN^{2L117}$ and *FRT82 Rheb*. To study the effects of *Rheb Tsc1* double mutants, recombinant chromosomes were generated with various alleles.

Gal4 under control of a heat-shock promoter (*hs-Gal4*) was used to ubiquitously overexpress *Rheb*. Second-instar larvae were transferred to tubes containing fresh squashed fly food and placed in a waterbath at 37 °C for 45 min. Then larvae were allowed to recover at room temperature for the indicated times before analysis in kinase assays.

Measurements and data analysis. The body weight of male flies three days after eclosion was measured with a precision scale (Mettler Toledo, Greifensee, Switzerland). Wings of female flies were mounted and analysed as previously described[10]. Scanning electron microscope (SEM) images of five female fly eyes per genotype were analysed to characterise the eye phenotypes. Rosettes of seven ommatidia were measured using the NIH image software to determine ommatidial area.

Extraction of larvae, kinase assays and western blotting. Larvae were extracted essentially as described[13], however with a modified extraction buffer (120 mM sodium chloride, 50 mM Tris-HCl at pH 7.0, 20 mM sodium fluoride, 1 mM benzamidine, 1 mM EDTA, 6 mM EGTA, 15 mM $Na_4P_2O_7$-$10H_2O$ and 1% NP-40; the following reagents were added shortly before use to the indicated concentrations: 1/5 volume Complete Mini protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland) prepared as a 5× stock in extraction buffer without the reagents listed hereafter, 2 mM AEBSF (Pefabloc SC (Roche)), 30 mM *para*-nitrophenylphosphate, 30 mM β-glycerolphosphate, 4 μM leupeptin, 2 μM aprotinin, 4 μM pepstatin and 100 μM phenyl methylsulphonyl fluoride).

Kinase activity assays of either S6K or dPKB were performed as described[13,22].

For immunoblotting, the following antibodies were used at the indicated dilutions: S6K D-20 antibody at 1:200 (ref. 24), dPKB antibody (ref. 34) at 1:1000 and α-tubulin antibody (Sigma, St Louis, MO) at 1:1000. HRP-conjugated secondary antibodies (Dako A/S, Glostrup, Denmark) were diluted 1:2000. Signals were detected using ECL western blotting detection reagents (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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1. Potter, C. J. & Xu, T. Mechanisms of size control. *Curr. Opin. Genet. Dev.* **11,** 279–286 (2001).
2. Johnston, L. A. & Gallant, P. Control of growth and organ size in *Drosophila*. *Bioessays* **24,** 54–64 (2002).

3. Marygold, S. & Leevers, S. Growth signaling: TSC takes its place. *Curr. Biol.* 12, R785–R787 (2002).
4. McManus, E. J. & Alessi, D. R. TSC1–TSC2: a complex tale of PKB-mediated S6K regulation. *Nature Cell Biol.* 4, E214–E216 (2002).
5. Radimerski, T., Montagne, J., Hemmings-Mieszczak, M. & Thomas, G. Lethality of *Drosophila* lacking TSC tumor suppressor function rescued by reducing dS6K signaling. *Genes Dev.* **16,** 2627–2632 (2002).
6. Oldham, S. & Hafen, E. Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends Cell Biol.* **13,** 79–85 (2003).
7. Leslie, N. R. & Downes, C. P. PTEN: The down side of PI 3-kinase signalling. *Cell. Signal.* **14,** 285–295 (2002).
8. Wienecke, R., Konig, A. & DeClue, J. E. Identification of tuberin, the tuberous sclerosis-2 product. Tuberin possesses specific Rap1GAP activity. *J. Biol. Chem.* **270,** 16409–16414 (1995).
9. Xiao, G. H., Shoarinejad, F., Jin, F., Golemis, E. A. & Yeung, R. S. The tuberous sclerosis 2 gene product, tuberin, functions as a Rab5 GTPase activating protein (GAP) in modulating endocytosis. *J. Biol. Chem.* **272,** 6097–6100 (1997).
10. Böhni, R. *et al.* Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4. *Cell* **97,** 865–875 (1999).
11. Brogiolo, W. *et al.* An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Curr. Biol.* **11,** 213–221 (2001).
12. Yamagata, K. *et al. rheb*, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein. *J. Biol. Chem.* **269,** 16333–16339 (1994).
13. Oldham, S., Montagne, J., Radimerski, T., Thomas, G. & Hafen, E. Genetic and biochemical characterization of dTOR, the *Drosophila* homolog of the target of rapamycin. *Genes Dev.* **14,** 2689–2694 (2000).
14. Zhang, H., Stallock, J. P., Ng, J. C., Reinhard, C. & Neufeld, T. P. Regulation of cellular growth by the *Drosophila* target of rapamycin dTOR. *Genes Dev.* **14,** 2712–2724 (2000).
15. Gao, X. *et al.* Tsc tumour suppressor proteins antagonize amino-acid-Tor signaling. *Nature Cell Biol.* **4,** 699–704 (2002).
16. Britton, J. S., Lockwood, W. K., Li, L., Cohen, S. M. & Edgar, B. A. *Drosophila*'s insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. *Dev. Cell* **2,** 239–249 (2002).
17. Tapon, N., Ito, N., Dickson, B. J., Treisman, J. E. & Hariharan, I. K. The *Drosophila* tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* **105,** 345–355 (2001).
18. Potter, C. J., Huang, H. & Xu, T. *Drosophila* Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. *Cell* **105,** 357–368 (2001).
19. Gao, X., Neufeld, T. P. & Pan, D. *Drosophila* PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways. *Dev. Biol.* **221,** 404–418 (2000).
20. Huang, H. *et al.* PTEN affects cell size, cell proliferation and apoptosis during *Drosophila* eye development. *Development* **126,** 5365–5372 (1999).
21. Gao, X. & Pan, D. TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. *Genes Dev.* **15,** 1383–1392 (2001).
22. Radimerski, T. *et al.* dS6K regulated cell growth is dPKB/dPI3K independent, but requires dPDK1. *Nature Cell Biol.* **4,** 251–255 (2002).
23. Haruta, T. *et al.* A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. *Mol. Endocrinol.* **14,** 783–794 (2000).
24. Montagne, J. *et al. Drosophila* S6 Kinase: A Regulator of Cell Size. *Science* **285,** 2126–2129 (1999).
25. Mach, K. E., Furge, K. A. & Albright, C. F. Loss of Rhb1, a Rheb-related GTPase in fission yeast, causes growth arrest with a terminal phenotype similar to that caused by nitrogen starvation. *Genetics* **155,** 611–622 (2000).
26. Newsome, T. P., Asling, B. & Dickson, B. J. Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics. *Development* **127,** 851–860 (2000).
27. Nairz, K., Stocker, H., Schindelholz, B. & Hafen, E. High-resolution SNP mapping by denaturing HPLC. *Proc. Natl Acad. Sci. USA* **99,** 10575–10580 (2002).
28. Hay, B. A., Wassarman, D. A. & Rubin, G. M. *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* **83,** 1253–1262 (1995).
29. Staveley, B. E. *et al.* Genetic analysis of protein kinase B (AKT) in *Drosophila*. *Curr. Biol.* **8,** 599–602 (1998).
30. Stocker, H. *et al.* Living with lethal concentrations of PIP3: A mutation in the PH domain of Akt/PKB restores the viability of flies lacking the tumor suppressor PTEN. *Science* **295,** 2088–2091 (2002).
31. Oldham, S. *et al.* The *Drosophila* insulin/IGF receptor controls growth and size by modulating PtdInsP3 levels. *Development* **129,** 4103–4109 (2002).
32. Neufeld, T. P., Delacruz, A. F. A., Johnston, L. A. & Edgar, B. A. Coordination of Growth and Cell Division in the *Drosophila* Wing. *Cell* **93,** 1183–1193 (1998).
33. Rintelen, F., Stocker, H., Thomas, G. & Hafen, E. PDK1 regulates growth through PKB and S6K in *Drosophila*. *Proc. Natl Acad. Sci. USA* **98,** 15020–15025 (2001).
34. Andjelkovic, M. *et al.* Developmental regulation of expression and activity of multiple forms of the *Drosophila* RAC protein kinase. *J. Biol. Chem.* **270,** 4066–4075 (1995).
35. Potter, C. J., Pedraza, L. & Xu, T. Akt regulates growth by directly phosphorylating Tsc2. *Nature Cell Biol.* **4,** 658–665 (2002).

Living With Lethal PIP3 Levels:
Viability of Flies Lacking PTEN Restored by a PH Domain Mutation in Akt/PKB

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14. C. Baugh, D. Grate, C. Wilson, *J. Mol. Biol.* **301**, 117 (2000).
15. W. A. Hendrickson, *Science* **254**, 51 (1991).
16. M. Costa, E. L. Christian, F. Michel, *RNA* **4**, 1055 (1998).
17. B. B. Konforti *et al.*, *Mol. Cell.* **1**, 433 (1998).
18. L. Zhang, J. Doudna, unpublished observations.
19. J. H. Cate *et al.*, *Science* **273**, 1678 (1996).
20. E. J. Sontheimer, P. M. Gordon, J. A. Piccirilli, *Genes Dev.* **13**, 1729 (1999).
21. A. M. Pyle, *Science* **261**, 709 (1993).
22. R. K. Sigel, A. Vaidya, A. M. Pyle, *Nature Struct. Biol.* **7**, 1111 (2000).
23. P. M. Gordon, J. A. Piccirilli, *Nature Struct. Biol.* **8**, 893 (2001).
24. R. K. Sigel, A. M. Pyle, personal communication.
25. J. H. Cate, R. L. Hanna, J. A. Doudna, *Nature Struct. Biol.* **4**, 553 (1997).
26. D. L. Abramovitz, R. A. Friedman, A. M. Pyle, *Science* **271**, 1410 (1996).
27. G. Chanfreau, A. Jacquier, *Science* **266**, 1383 (1994).
28. S. L. Yean, G. Wuenschell, J. Termini, R. J. Lin, *Nature* **408**, 881 (2000).
29. P. Z. Qin, A. M. Pyle, *Curr. Opin. Struct. Biol.* **8**, 301 (1998).
30. V. T. Chu, Q. Liu, M. Podar, P. S. Perlman, A. M. Pyle, *RNA* **4**, 1186 (1998).
31. V. T. Chu, C. Adamidi, Q. Liu, P. S. Perlman, A. M. Pyle, *EMBO J.* **20**, 6866 (2001).
32. J. A. Berglund, M. Rosbash, S. C. Schultz, *RNA* **7**, 682 (2001).
33. J. Wu, J. L. Manley, *Genes Dev.* **3**, 1553 (1989).
34. Y. Zhuang, A. M. Weiner, *Genes Dev.* **3**, 1545 (1989).
35. C. C. Query, M. J. Moore, P. A. Sharp, *Genes Dev.* **8**, 587 (1994).
36. R. Parker, P. G. Siliciano, C. Guthrie, *Cell* **49**, 229 (1987).
37. M. Carson, *J. Appl. Crystallogr.* **24**, 958 (1991).
38. D. L. Daniels, W. J. Michels, A. M. Pyle, *J. Mol. Biol.* **256**, 31 (1996).
39. A. R. Ferre-D'Amare, K. Zhou, J. A. Doudna, *Nature* **395**, 567 (1998).

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Living with Lethal PIP3 Levels: Viability of Flies Lacking PTEN Restored by a PH Domain Mutation in Akt/PKB

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The phosphoinositide phosphatase PTEN is mutated in many human cancers. Although the role of PTEN has been studied extensively, the relative contributions of its numerous potential downstream effectors to deregulated growth and tumorigenesis remain uncertain. We provide genetic evidence in *Drosophila melanogaster* for the paramount importance of the protein kinase Akt [also called protein kinase B (PKB)] in mediating the effects of increased phosphatidylinositol 3,4,5-trisphosphate (PIP3) concentrations that are caused by the loss of PTEN function. A mutation in the pleckstrin homology (PH) domain of Akt that reduces its affinity for PIP3 sufficed to rescue the lethality of flies devoid of PTEN activity. Thus, Akt appears to be the only critical target activated by increased PIP3 concentrations in *Drosophila*.

Mutations in the tumor suppressor gene *PTEN* (the phosphatase and tensin homolog on chromosome 10) are frequent in glioblastomas, endometrial carcinoma, melanomas, and prostate cancer (*1*). Furthermore, two dominant hamartoma syndromes, Cowden disease and Bannayan-Zonana syndrome, are linked to germ line mutations in *PTEN* (*1*). The PTEN protein carries a phosphatase domain resembling those of dual-specificity protein phosphatases (*2–4*). Although it can dephosphorylate protein substrates such as focal adhesion kinase (*5*) and the adapter protein Shc (*6*), PTEN's predominant enzymatic activity appears to be the dephosphorylation of phosphoinositides at the D3 position. Because PTEN uses the second messenger PIP3 as a substrate, PTEN antagonizes the function of phosphatidylinositol-3 kinase (PI3K) (*7*, *8*). Immortalized mouse embryonic fibroblasts or embryonic stem cells lacking PTEN function show an approximately twofold increase in PIP3 concentrations (*9*, *10*). PIP3 interacts with a wide variety of PH domain-containing proteins, including the serine-threonine kinases Akt (also called PKB) and phosphoinositide-dependent kinase 1 (PDK1), Btk family tyrosine kinases, guanine nucleotide exchange factors for the Rho and Arf families of small guanosine triphosphatases, and phospholipase Cγ (*11*, *12*). The plethora of proteins that are potentially regulated by PIP3 provides widespread signaling potential for this lipid second messenger.

Genetic analyses in model organisms have implicated PTEN as a negative regulator of insulin receptor signaling. In the nematode *Caenorhabditis elegans*, PTEN antagonizes the activity of the PI3K AGE-1 in the regulation of metabolism, development, and life span (*13–16*). In the fruit fly *Drosophila melanogaster*, PTEN counteracts signaling downstream of the insulin receptor to control cellular growth (*17–19*). There are, however,

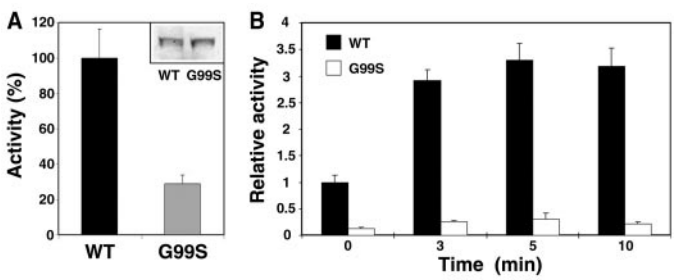

Fig. 1. Reduced kinase activity caused by an amino acid substitution in the PH domain of dAkt. (**A**) Effect of the G99S substitution in the PH domain on dAkt kinase activity from larval extracts (*42*). Activity from wild-type larvae was considered to be 100%. Inset, dAkt protein was detected in 40 μg of larval extracts using the same antiserum. (**B**) Reduced insulin-induced activation of the G99S mutant dAkt. The dAkt constructs were expressed in HEK 293 cells (*43*). Transfected cells were starved for 24 hours before stimulation with insulin for the indicated time periods, and dAkt kinase activity was determined (*44*). The activity of wild-type dAkt from unstimulated cells was considered to be relative activity = 1.

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additional phenotypes associated with mutations in *PTEN* that cannot easily be reconciled with an exclusive function of PTEN in insulin receptor signaling [for example, the burst vulva phenotype in *C. elegans* (*13*) and defects in the actin cytoskeleton in *Drosophila* (*17*)]. To better understand the consequences of loss of PTEN function, it would be useful to know which important downstream effectors react to increased PIP3 concentrations and whether PTEN has other physiological substrates in addition to PIP3.

The protein kinase Akt is an important component of insulin receptor signaling (*20*). Akt is recruited to the plasma membrane by virtue of the interaction of its NH_2–terminally located PH domain with PIP3. At the membrane, subsequent phosphorylation events by PDK1 and an unidentified kinase lead to the full activation of Akt (*21*–*23*). In *PTEN*-deficient mouse embryonic fibroblasts and embryonic stem cells, Akt is phosphorylated and activated (*9*, *10*). The phenotypes associated with *Akt* mutations in both *C. elegans* and *Drosophila* are consistent with its role in signal transduction downstream of the insulin receptor (*24*–*27*).

We monitored three properties of *Drosophila* Akt (dAkt) separately: kinase activity, abundance of the protein, and membrane localization. We relied entirely on mutations in the endogenous gene encoding dAkt to avoid potential side effects caused by overexpression of mutant proteins. $dAkt^{1}$ encodes a catalytically inactive protein, dAktF327I (*25*). The viable $dAkt^{4226}$ allele contains a P-element insertion upstream of the *dAkt* gene and therefore results in the reduced expression of wild-type dAkt protein (*19*, *28*). Finally, we characterized the viable hypomorphic mutation $dAkt^{3}$ (*29*) that selectively impairs the membrane recruitment of dAkt in response to increased concentrations of PIP3. Sequencing of genomic DNA extracted from $dAkt^{3}$ homozygous flies revealed a single nucleotide exchange resulting in the substitution of a serine residue for a nonconserved glycine at the end of the sixth β sheet of the PH domain. To address the mechanism by which this $Gly^{99} \rightarrow Ser^{99}$ (G99S) mutation in the PH domain affects dAkt, we compared the amount of dAkt protein and activity in wild-type and $dAkt^{3}$ mutant larvae. Whereas no apparent difference in expression of the protein was observed (Fig. 1A, inset), dAkt activity from the mutant larvae represented only 30% of that in wild-type larval extracts (Fig. 1A). We also expressed epitope-tagged forms of wild-type dAkt, catalytically inactive dAktF327I, and PH domain mutant dAktG99S in insulin-responsive human embryonic kidney (HEK) 293 cells. All three proteins were expressed in similar amounts (*30*). dAktG99S activity from insulin-stimulated cells was reduced by about 90% as compared to that of the wild-type kinase (Fig. 1B). All forms of dAkt proteins were detected in the cytosol of unstimulated cells (Fig. 2, A, D, and G). Stimulation of the cells with insulin for 5 min resulted in association of the wild-type and the catalytically inactive enzymes with the plasma membrane, but failed to recruit the dAktG99S mutant protein (Fig. 2, B, E, and H). In contrast, treatment of HEK 293 cells with the protein-tyrosine phosphatase inhibitor pervanadate, a potent activator of Akt (*31*), led to membrane recruitment of all dAkt proteins (Fig. 2, C, F, and J).

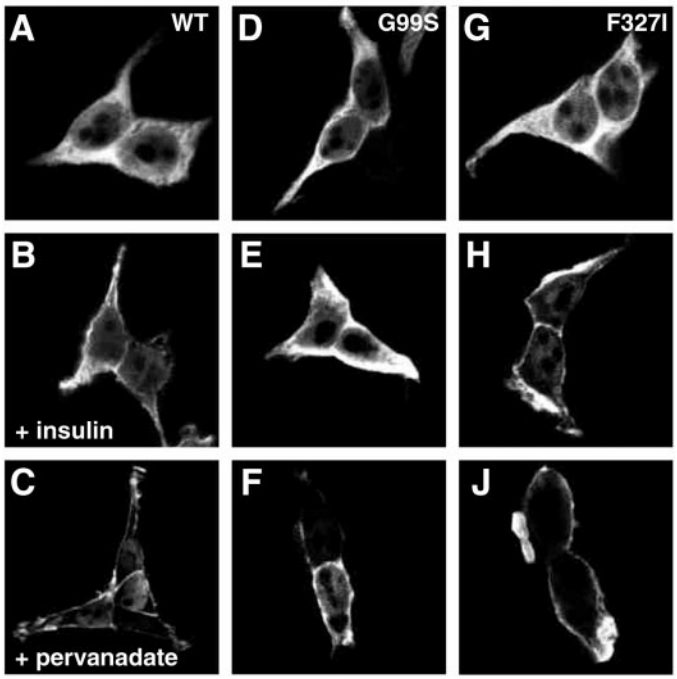

Fig. 2. Reduced membrane localization of the G99S mutation of dAkt. HEK 293 cells plated on coverslips were transfected with epitope-tagged wild-type (**A** to **C**), G99S (**D** to **F**), and F327I (**G** to **J**) dAkt and deprived of serum for 16 hours before stimulation with insulin (B, E, H) or pervanadate (C, F, J) for 5 min. Fixed and permeabilized cells were incubated first with the monoclonal antibody 12CA5 to the HA epitope and then with fluorescein isothiocyanate–conjugated secondary antibody. An analysis by confocal microscopy revealed the subcellular localization of the dAkt variants.

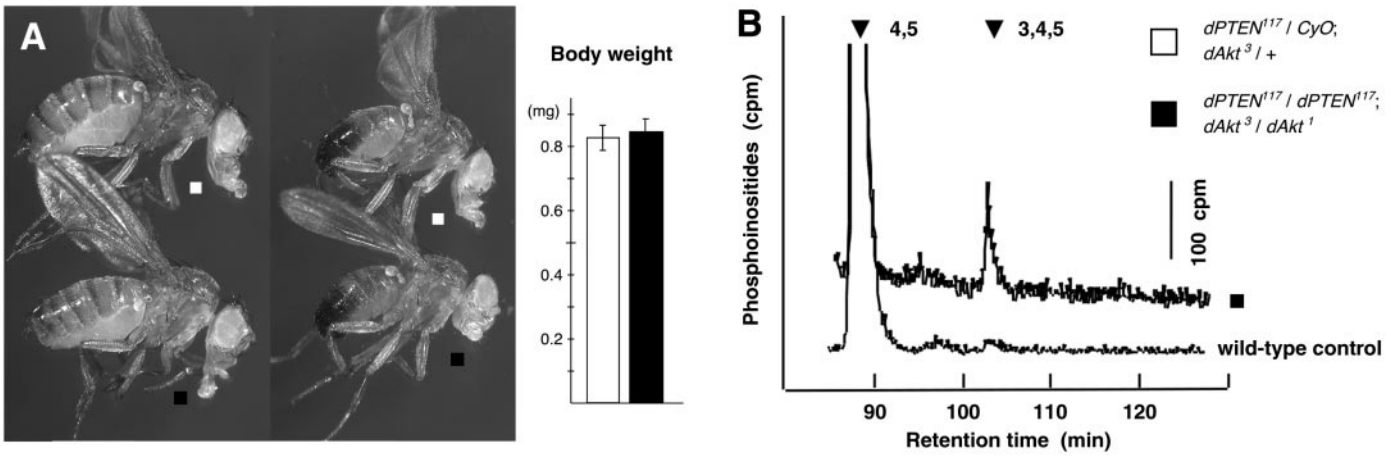

Fig. 3. Restored viability of flies lacking dPTEN function by the PH domain mutation in dAkt. (**A**) Morphology and weight of *dPTEN* mutant flies rescued by $dAkt^{3}/dAkt^{1}$. The left panel shows female flies, the middle panel shows male flies, and the right panel shows the weight of adult male flies. (**B**) PIP3 concentrations in flies devoid of dPTEN function rescued by the $dAkt^{3}$ mutation.

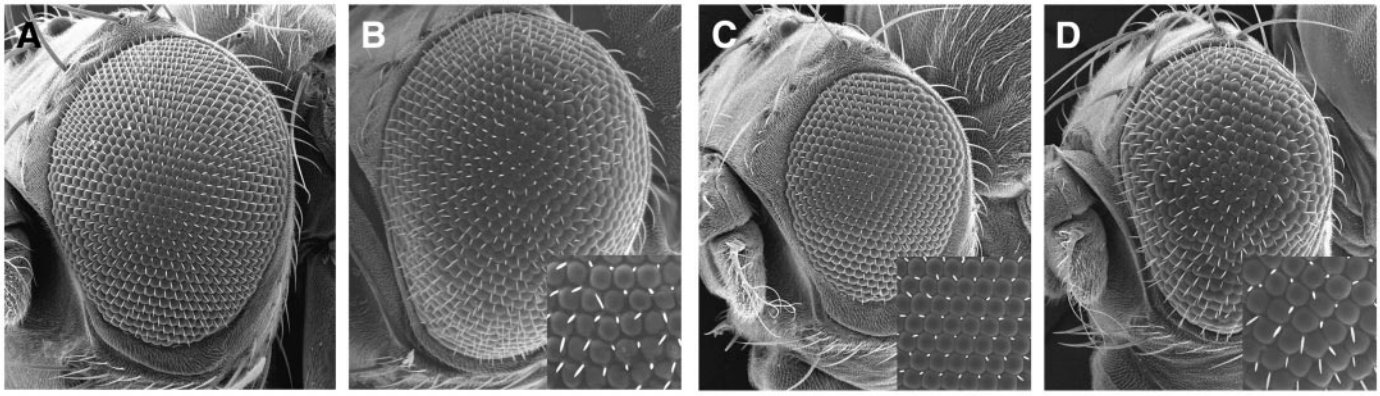

Fig. 4. Growth in the developing *Drosophila* eye promoted by activated dAkt. (**A** to **D**) Scanning electron micrographs of compound eyes of female flies; the anterior is to the left. (A) Wild type. (B) Overexpressing a membrane-tethered version of dAkt (*GMR-Gal4 UAS-myr-dAkt*). (C) *chico* mutant. (D) *GMR-Gal4 UAS-myr-dAkt* in a *chico* mutant background (*45*).

Consistently, pervanadate treatment stimulated dAktG99S activity to 80% of the wild-type level. However, pervanadate-induced activation of the mutant protein occurred more slowly than did that of the wild-type kinase (*30*). Taken together, these data indicate that the G99S substitution reduces the association of dAkt with the plasma membrane, probably by affecting the affinity of its PH domain for PIP3. Thus, $dAkt^{3}$ enabled us to study the consequences of impaired recruitment of dAkt to the plasma membrane.

We combined the *dAkt* alleles with null mutations in *dPTEN* (*32*). Animals lacking dPTEN function die during larval stages. A reduction in *dAkt* expression using the viable $dAkt^{4226}$ allele did not rescue the lethality associated with *dPTEN*. Similarly, animals doubly mutant for *dPTEN* and $dAkt^{1}$ did not survive. Thus, either dAkt activation is not the sole reason for the lethality caused by loss of PTEN, or dAkt function is not dispensable in the absence of dPTEN. The latter hypothesis is strongly supported by results obtained with the *dAkt* allele that selectively impairs the membrane recruitment of dAkt. Flies devoid of functional dPTEN were rescued to viability by any *dAkt* allelic combination that included $dAkt^{3}$ (Fig. 3A) (*33*). The rescued flies did not display morphological defects that would be expected in light of the phenotypes ascribed to clones of *dPTEN* mutant cells (*17*). Tangential sections through compound eyes revealed essentially normal ommatidial and rhabdomeric structures, and the wings of the rescued flies showed no abnormalities in the venation, such as missing crossveins (*34*). We determined the PIP3/PIP2 ratio by metabolic labeling of phospholipids from larvae (*35*). PIP3 concentrations were increased in the *dPTEN dAkt* doubly mutant larvae as compared to those of wild-type larvae (Fig. 3B), excluding the possibility that PIP3 concentrations remain within physiological limits by a feedback regulation mechanism involving dAkt. This suggests that the potential activation of a number of PH domain–containing proteins other than dAkt does not interfere with viability.

Our results indicate that the activation of dAkt is the only crucial outcome of the loss of dPTEN function. Activation of dAkt should therefore mimic the dPTEN loss-of-function phenotype. We expressed a constitutively activated membrane-anchored dAkt during eye development (*36*). The resulting eyes were increased in size due to enlarged ommatidia (Fig. 4B), a phenotype similar to that seen in clones of *dPTEN* mutant cells (*17–19*). This overgrowth phenotype is independent of upstream signals, because it was still evident in a *chico* or *Dp110/PI3K* mutant background (Fig. 4D) (*34*).

We conclude that flies devoid of the tumor suppressor dPTEN can live with abnormally high concentrations of PIP3 if only the affinity of dAkt for PIP3 is decreased. Thus, the PH domain–mediated translocation of dAkt to the membrane and its subsequent activation is the only lethal event triggered by increased PIP3 concentrations. Because the PH domain of Akt interacts with the substrate of PTEN's lipid phosphatase activity, we also conclude that PTEN does not exert any essential function other than the dephosphorylation of PIP3.

References and Notes

1. L. Simpson, R. Parsons, *Exp. Cell Res.* **264**, 29 (2001).
2. J. Li *et al.*, *Science* **275**, 1943 (1997).
3. P. A. Steck *et al.*, *Nature Genet.* **15**, 356 (1997).
4. D. M. Li, H. Sun, *Cancer Res.* **57**, 2124 (1997).
5. M. Tamura *et al.*, *Science* **280**, 1614 (1998).
6. J. Gu *et al.*, *J. Cell Biol.* **146**, 389 (1999).
7. T. Maehama, J. E. Dixon, *J. Biol. Chem.* **273**, 13375 (1998).
8. _____, *Trends Cell Biol.* **9**, 125 (1999).
9. V. Stambolic *et al.*, *Cell* **95**, 29 (1998).
10. H. Sun *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6199 (1999).
11. B. Vanhaesebroeck *et al.*, *Annu. Rev. Biochem.* **70**, 535 (2001).
12. S. J. Leevers, B. Vanhaesebroeck, M. D. Waterfield, *Curr. Opin. Cell Biol.* **11**, 219 (1999).
13. S. Ogg, G. Ruvkun, *Mol. Cell* **2**, 887 (1998).
14. E. B. Gil, E. Malone Link, L. X. Liu, C. D. Johnson, J. A. Lees, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 2925 (1999).
15. V. T. Mihaylova, C. Z. Borland, L. Manjarrez, M. J. Stern, H. Sun, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 7427 (1999).
16. J. P. Rouault *et al.*, *Curr. Biol.* **9**, 329 (1999).
17. D. C. I. Goberdhan, N. Paricio, E. C. Goodman, M. Mlodzik, C. Wilson, *Genes Dev.* **13**, 3244 (1999).
18. H. Huang *et al.*, *Development* **126**, 5365 (1999).
19. X. Gao, T. P. Neufeld, D. Pan, *Dev. Biol.* **221**, 404 (2000).
20. M. A. Lawlor, D. R. Alessi, *J. Cell Sci.* **114**, 2903 (2001).
21. J. Downward, *Curr. Opin. Cell Biol.* **10**, 262 (1998).
22. P. J. Coffer, J. Jin, J. R. Woodgett, *Biochem. J.* **335**, 1 (1998).
23. T. O. Chan, S. E. Rittenhouse, P. N. Tsichlis, *Annu. Rev. Biochem.* **68**, 965 (1999).
24. S. Paradis, G. Ruvkun, *Genes Dev.* **12**, 2488 (1998).
25. B. E. Staveley *et al.*, *Curr. Biol.* **8**, 599 (1998).
26. J. Verdu, M. A. Buratovich, E. L. Wilder, M. J. Birnbaum, *Nature Cell Biol.* **1**, 500 (1999).
27. S. E. Scanga *et al.*, *Oncogene* **19**, 3971 (2000).
28. A. C. Spradling *et al.*, *Genetics* **153**, 135 (1999).
29. The $dAkt^{3}$ allele was found in a collection of homozygous viable mutations from Ch. Zuker and E. Koundakjian (University of California, San Diego) because it yields flies of severely reduced body size.
30. M. Andjelkovic, B. A. Hemmings, unpublished results.
31. M. Andjelkovic *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5699 (1996).
32. The *dPTEN* alleles used are $dPTEN^{dj189}$ (*19*) and $dPTEN^{117}$ (*37*). Both are presumably null alleles. We also tested them over a deficiency lacking the *dPTEN* locus, *Df(2L)170B*. Because this deficiency also lacks *chico*, an upstream component of insulin receptor signaling, we reintroduced *chico* by means of a genomic rescue construct. In all cases, we obtained similar results.
33. Whereas the strongest allelic combination $dAkt^{1}/dAkt^{3}$ completely rescued the *dPTEN* mutant flies to wild-type size, the combinations $dAkt^{3}/dAkt^{3}$ and $dAkt^{3}/dAkt^{4226}$ could rescue the lethality associated with loss of PTEN function, but the resulting flies were slightly enlarged. Furthermore, we observed a variability of the phenotypes in all combinations. Consistently, flies that emerged earlier showed a tendency to be of increased size, whereas some retarded flies were of smaller size.
34. H. Stocker, E. Hafen, unpublished results.
35. Nonwandering third instar larvae were phosphate starved in phosphate-free Schneider S2 medium and then labeled with 2 mCi per sample of inorganic ^{32}P (50 mCi/ml). PIP3 and PIP2 levels were determined according to (*38*).
36. To anchor dAkt to the plasma membrane, the myristoylation-palmitoylation motif from the Lck tyrosine kinase was fused to the NH_2-terminus of hemagglutinin (HA) epitope–tagged dAkt, as previously described for mammalian Akt (*39*). Analysis of the subcellular localization by immunofluorescence using the epitope antibody to HA confirmed the constitutive membrane localization of myr-dAkt.

37. S. Oldham, E. Hafen, unpublished results.
38. A. Arcaro, M. P. Wymann, *Biochem. J.* **296**, 297 (1993).
39. M. Andjelkovic *et al.*, *J. Biol. Chem.* **272**, 31515 (1997).
40. M. Andjelkovic *et al.*, *J. Biol. Chem.* **270**, 4066 (1995).
41. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; E, Glu; F, phe; G, Gly; I, Ile; P, Pro; R, Arg; S, Ser; and T, Thr.
42. dAkt was immunoprecipitated from wild-type and *dAkt*^{3} larvae with a rabbit polyclonal antibody raised against recombinant dAkt-66 (*40*). In vitro kinase assays were performed, as described, using the peptide GRPRTSSAEG (*41*) as a substrate (*39*).
43. HEK 293 cells were transfected by a modified calcium phosphate method, as described (*39*). The transfection mixture was removed after a 16-hour incubation, and cells were serum-starved for 24 hours before stimulation with 0.5 to 1 μM insulin (Boehringer Mannheim).
44. The HA epitope–tagged dAkt proteins were immunoprecipitated from 100 μg of cell-free extracts using the monoclonal antibody 12CA5 coupled to protein A–Sepharose. In vitro kinase assays were performed, as described (*39*).
45. The size difference of the eyes in Fig. 4, B and D, is entirely due to varying numbers of ommatidia. The size of the ommatidia, however, is comparable (insets) and massively larger than in *chico* mutants (inset in 4C). The failure of myr-dAkt to compensate for the reduced number of ommatidia in *chico* mutants is consistent with the late onset of expression driven by *GMR-Gal4*.
46. We thank Ch. Zuker and E. Koundakjian for the *dAkt*^{3} allele; T. Radimerski for the S2 labeling medium; T. Gutjahr, Ch. Hugentobler, R. Bopp, P. Zipperlen, P. Cron, P. Müller, and H. Angliker for technical support; K. Basler and P. Gallant for critical reading of the manuscript and valuable suggestions; and D. Pan and S. Leevers for providing fly stocks. Supported by grants from the Schweizerische Krebsliga (B.A.H. and M.P.W.) and the Swiss National Science Foundation (E.H.).

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Reverse Transcriptase–Mediated Tropism Switching in *Bordetella* Bacteriophage

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Host-pathogen interactions are often driven by mechanisms that promote genetic variability. We have identified a group of temperate bacteriophages that generate diversity in a gene, designated *mtd* (major tropism determinant), which specifies tropism for receptor molecules on host *Bordetella* species. Tropism switching is the result of a template-dependent, reverse transcriptase–mediated process that introduces nucleotide substitutions at defined locations within *mtd*. This cassette-based mechanism is capable of providing a vast repertoire of potential ligand-receptor interactions.

The infectious cycles of *Bordetella* subspecies, which cause respiratory infections in humans and other mammals, is controlled by the BvgAS signal transduction system (*1*). Using a multistep phosphorelay, the BvgS transmembrane sensor-kinase and the BvgA transcriptional regulator couple environmental signals to expression of cell surface and secreted molecules (*1*, *2*). The Bvg$^+$ phase, which is necessary and sufficient for respiratory tract colonization, is characterized by a high level of BvgAS activity and expression of virulence and colonization factors that include adhesins, toxins, and a type III secretion system (*2*). In the Bvg$^-$ phase, BvgAS is inactive, virulence and colonization factors are not expressed, and numerous genes are induced, including motility loci in *Bordetella bronchiseptica* and virulence repressed genes in *Bordetella pertussis* (*2*, *3*). The Bvg$^-$ phase appears to be adapted to ex vivo growth and survival in *B. bronchiseptica* (*4*). Recent evidence suggests that BvgAS is capable of controlling a spectrum of distinct phenotypic phases in response to subtle changes in signal intensity (*5*, *6*).

In a search for generalized transducing vectors, we identified several temperate bacteriophages present in clinical isolates of *B. bronchiseptica* that displayed a marked tropism for Bvg$^+$ as opposed to Bvg$^-$ phase bacteria. The efficiency of plaque formation of a representative phage, designated BPP-1 (Bvg plus tropic phage–1), was 10^6-fold higher on Bvg$^+$ phase RB50 (wild-type *B. bronchiseptica*) than on an isogenic Bvg$^-$ phase-locked strain (Δ*bvgS*) (Fig. 1A). An adsorption assay (Fig. 1B) indicated that the BPP-1 receptor is specifically expressed in the Bvg$^+$ phase. Mutagenesis of loci encoding Bvg$^+$ phase surface factors showed that deletion of *prn*, which encodes the adhesin pertactin (*7*), eliminated BPP-1 adsorption and decreased phage plaquing to a level similar to that observed on Bvg$^-$ phase cells (Fig. 1, A and B). Ectopic expression of *prn*

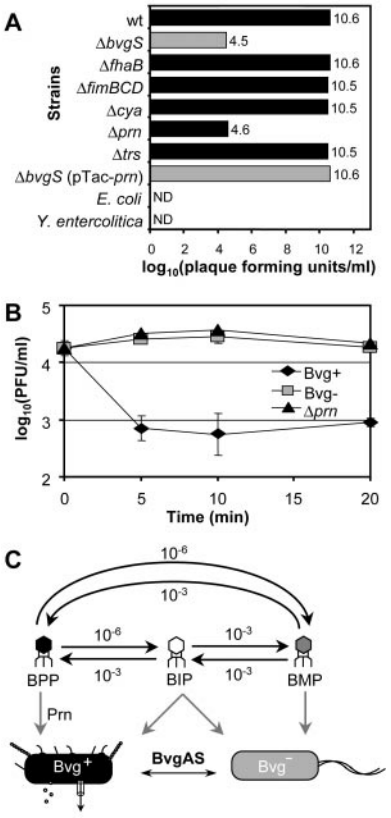

Fig. 1. (**A**) Efficiency of plaquing by a high-titer BPP-1 lysate [4×10^{10} plaque forming units/ml (pfu/ml)] on isogenic *B. bronchiseptica* mutants. The Δ*bvgS*, Δ*fhaB*, Δ*fimBCD*, Δ*cyaA*, Δ*prn*, and Δ*trs* mutations are in-frame deletion mutations that eliminate BvgS activity or expression of FHA (filamentous hemagglutinin), fimbriae, adenylate cyclase toxin, pertactin, or the type III secretion apparatus. Black bars indicate Bvg$^+$ phase strains; gray bars indicate strains genetically locked in the Bvg$^-$ phase. pTac-prn is a complementing plasmid that expresses pertactin under control of the Tac promoter. *E. coli* strain DH5α and *Yersinia enterocolitica* strain JB580v did not support phage growth. ND, no plaque detected. (**B**) BPP-1 adsorption assay (*8*) with the use of wild-type *B. bronchiseptica* strain RB50 (*4*), isogenic Bvg$^-$ (Δ*bvgS*), and Δ*prn* derivatives, all grown under Bvg$^+$ phase conditions. (**C**) Summary of tropism switching frequencies by *Bordetella* phages. A spontaneous mutant resistant to BIP-1 but still sensitive to BPP-1 and BMP-1 were used to obtain switching frequencies from BIP-1 to BPP or BMP tropic variants.

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In the following sections, some studies that are relevant to the topic of insulin signaling and growth control and to which I contributed significantly will be presented.

An evolutionarily conserved function of the *Drosophila* insulin receptor and of insulin-like peptides in growth control

This study describes the identification of the seven insulin-like peptides (termed DILPs) encoded by the *Drosophila* genome. Overexpression of one of these peptides, DILP2, results in a growth promotion, and this effect is dependent on an intact insulin signal transduction cascade.

PDK1 regulates growth through Akt and S6K in *Drosophila*

A genetic characterization of phosphoinositide-dependent kinase 1 (PDK1), a serine/threonine kinase that phosphorylates PKB and S6K.

The *Drosophila* Forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling

FOXO, a member of the forkhead family of transcription factors, is identified as a putative target of PKB. Surprisingly, the loss of FOXO function does not result in overgrowth. However, the growth deficit of flies with impaired insulin signaling is ameliorated by the simultaneous loss of FOXO function.

Diet-dependent effects of the *Drosophila* Mnk1/Mnk2 homolog Lk6 on growth via eIF4E

Lk6, the *Drosophila* homolog of Mnk1/2 kinases, phosphorylates the translation initiation factor eIF4E. Whether eIF4E phosphorylation results in stimulation or inhibition of translation is under debate. This study demonstrates genetically that eIF4E phosphorylation by Lk6 promotes growth, but that Lk6 overexpression can exert a negative effect, depending on the composition of the food source.

An evolutionarily conserved function of the *Drosophila* insulin receptor and of insulin-like peptides in growth control

Walter Brogiolo, Hugo Stocker, Tomoatsu Ikeya, Felix Rintelen, Rafael Fernandez and Ernst Hafen

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An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control

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Background: Size regulation is fundamental in developing multicellular organisms and occurs through the control of cell number and cell size. Studies in *Drosophila* have identified an evolutionarily conserved signaling pathway that regulates organismal size and that includes the *Drosophila* insulin receptor substrate homolog Chico, the lipid kinase PI(3)K (Dp110), DAkt1/dPKB, and dS6K.

Results: We demonstrate that varying the activity of the *Drosophila* insulin receptor homolog (DInr) during development regulates organ size by changing cell size and cell number in a cell-autonomous manner. An amino acid substitution at the corresponding position in the kinase domain of the human and *Drosophila* insulin receptors causes severe growth retardation. Furthermore, we show that the *Drosophila* genome contains seven insulin-like genes that are expressed in a highly tissue- and stage-specific pattern. Overexpression of one of these insulin-like genes alters growth control in a DInr-dependent manner.

Conclusions: This study shows that the *Drosophila* insulin receptor autonomously controls cell and organ size, and that overexpression of a gene encoding an insulin-like peptide is sufficient to increase body size.

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Background

Each individual organ grows by controlling cell number and/or cell size to reach its final dimensions in relation to the size of the organism. This process is tightly regulated and modulated by environmental factors such as nutrient availability and temperature [1–4]. How organ growth is coordinated within a single individual is still poorly understood. In mammals, hormones and growth factors are known to play a predominant role in controlling organismal growth by orchestrating cell growth, cell proliferation, and cell survival [5, 6]. Reducing the levels of growth hormone or its mediators, IGF1 and the IGF1 receptor (IGF1R), strongly affects body and organ size. In contrast to the well-established role of the IGF1R in growth control, a corresponding role of the insulin receptor is less well understood.

Recently, genetic studies in *Drosophila* have highlighted a conserved signaling pathway that plays an essential role in controlling body, organ, and cell size. This pathway involves the homolog of the insulin receptor substrates (Chico), PI(3)K (Dp110), PTEN (dPTEN), Akt/PKB (DAkt1/dPKB), and S6K (dS6K). Mutations in any one of these components lead to a change in cell size and, with the exception of dS6K, in cell number as well [7–14]. Conversely, overexpression of Dp110 or DAkt1 leads to an increased cell size without affecting cell numbers [9, 13]. Thus, it appears that stimulation of the PI(3)K/PKB pathway alone is not sufficient to promote cell growth and cell cycle progression.

The *Drosophila* homolog of the insulin/IGF1 receptor, DInr, is essential for normal development and is required for the formation of the epidermis and the nervous system during embryogenesis [15]. All described alleles of *dinr* are recessive embryonic or early larval lethal. Only weak heteroallelic combinations of *dinr* alleles were found to be viable and yield adults with a severe developmental delay, small body size, and female sterility [15, 16]. It is not known, however, whether the effect of DInr on growth is cell autonomous and whether activation of DInr is sufficient to promote growth and cell division. Furthermore, the identity of a ligand(s) for DInr has remained elusive.

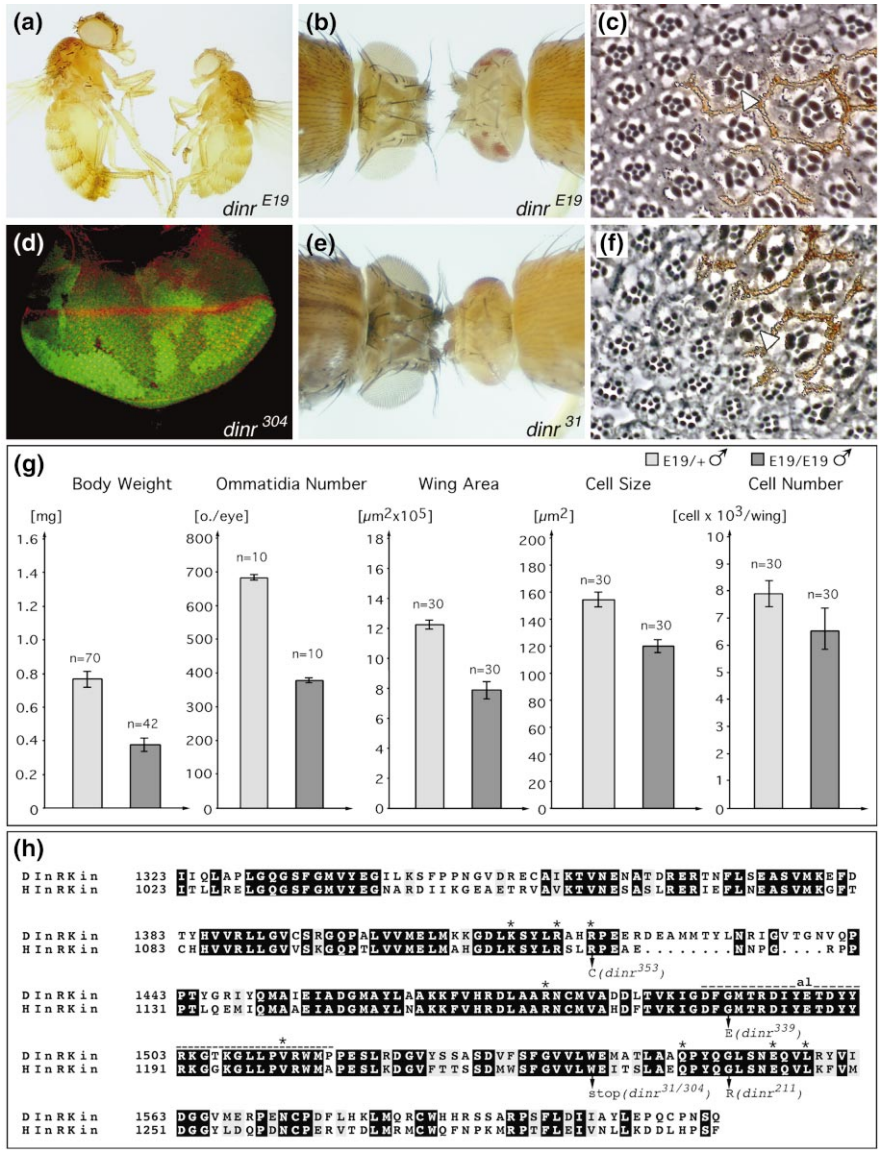
This study examines the effects on cell, organ, and organismal size when activity levels of DInr and of a new putative ligand are changed during development. Furthermore, we describe the structure and expression pattern during development of seven insulin-like genes in *Drosophila*.

Results and discussion

DInr regulates body and organ size by altering cell number and cell size in a cell-autonomous manner

Flies that are homozygous for a partial loss-of-function mutation in *dinr* (*dinr*^{E19}) [16] show a phenotype similar

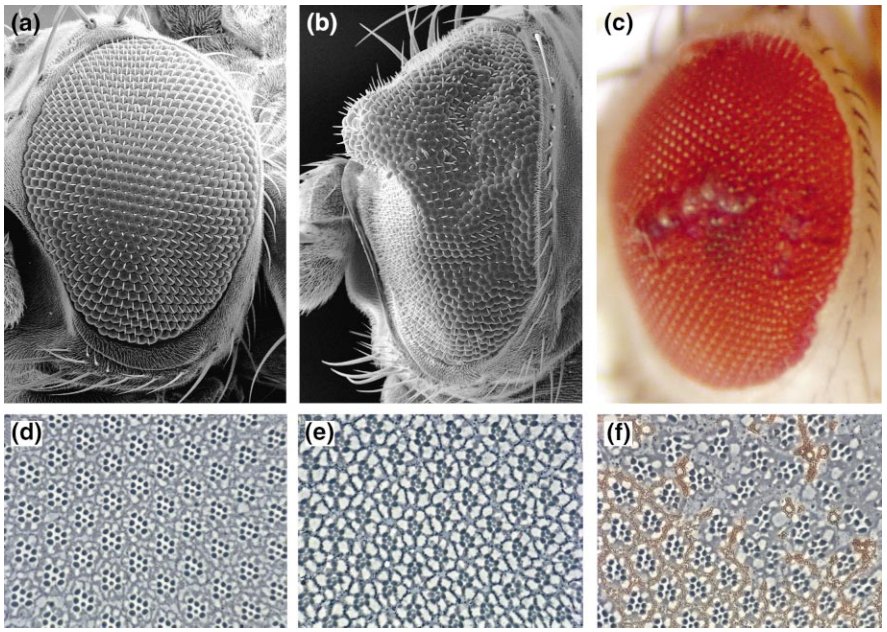
Figure 1



DInr regulates body and organ size by altering cell number and cell size in a cell-autonomous manner. **(a)** *dinr*^{E19}/*dinr*^{E19} flies (right) show a proportionate body size reduction compared to *dinr*^{E19}/+ control flies (left). **(b,e)** Selective removal of DInr function in eye progenitor cells generates flies with strongly reduced eyes and head capsule while all other body parts are of wild-type size. Note the relatively smaller head size of a null allele (e) than of a hypomorphic allele (b) compared to controls. **(c,f)** Tangential sections through a mosaic eye containing small homozygous *dinr* mutant ommatidia (lacking pigment) and normal-sized heterozygous ommatidia (containing pigment). Homozygous *dinr*^{E19} (c) and *dinr*^{31} (f) mutant photoreceptors are reduced in size by approximately one third and more than half, respectively. Arrowheads point to rhabdomeres of small homozygous mutant photoreceptors within genotypically mixed ommatidial units coexisting with normal heterozygous photoreceptors, demonstrating a cell-autonomous requirement for DInr. **(d)** Confocal microscope section of a third instar eye imaginal disc containing mitotic clones of *dinr*^{304} mutant cells (absence of green staining), which are significantly smaller than their wild-type (bright green staining) sister clones; anterior is to the top. **(g)** Quantitation of body and organ size in *dinr*^{E19} homozygous mutant male flies and in heterozygous siblings. In homozygous flies, the body weight and number of ommatidia is reduced by 52% and 45%, respectively. The wing area is decreased by 36%, due to a significant decrease in cell size and cell number by 23% and 17%, respectively. Values are means ± standard deviation; all values of homozygous flies are significantly reduced relative to their heterozygous siblings (t-test, $p < 0.0001$). Similar results were obtained with female flies. **(h)** Five *dinr* alleles carry point mutations in conserved amino acid residues of the kinase domain. An alignment of the kinase domain of the human insulin receptor (HInRKin) and the *Drosophila* homolog of insulin receptor (DInRKin) is shown with the localization and molecular description of the point mutations. *dinr*^{31} and *dinr*^{304} are nonsense mutations in the same codon at different nucleotide positions (Trp1439Stop). The mutations leading to an amino acid substitution are *dinr*^{353} (Arg1419Cys), *dinr*^{339} (Gly1491Glu), and *dinr*^{211} (Gly1551Arg). Asterisks denote predicted active site residues conferring substrate specificity [21]. The shading marks amino acid identity (black) or similarity (gray). We used the amino acid numbering of Fernandez *et al.* [15] for DInRKin and of Ebina *et al.* [45] for HInRKin. The sequences were aligned with the GCG program package. The abbreviations are single amino acid letter code, with "al" indicating an activation loop. Flies were of the following genotypes: (a) *y w; dinr*^{E19}*/TM3, Sb Ser* (left) and *y w; dinr*^{E19}*/dinr*^{E19} (right), (b,e) *y w ey-Flp; FRT82B dinr*^{E19,31}*/TM6B, y*$^{+}$ (left) and *y w ey-Flp; FRT82B dinr*^{E19,31}*/FRT82B P(w*$^{+}$*) 3R3.7* (right), (c,f) *y w ey-Flp; FRT82B dinr*^{E19,31}*/FRT82B P(w*$^{+}$*) 3R3.7*, (d) *y w hsFlp/y w; FRT82B dinr*^{304}*/FRT82B P(arm-lacZ, w*$^{+}$*)*, and (g) *y w; dinr*^{E19}*/+ and y w; dinr*^{E19}*/dinr*^{E19}.

to that previously described for weak heteroallelic combinations. The developmental time is extended from 10 to 20 days, and body size is severely but proportionally reduced. The mutant flies are approximately half the weight of their heterozygous siblings (Figure 1a,g), and females are sterile. Furthermore, like *chico* mutant flies, *dinr*^{E19} flies have an almost 2-fold increase in lipid content (data not shown). The small body size is attributable to a reduction in cell size and cell number by 23% and 17%, respectively (Figure 1g) as revealed by measuring cell density in the wing. Similarly, the average number of ommatidia in the compound eye of mutant male flies is

Figure 2



Overexpression of DInr in the eye leads to a hyperproliferative outgrowth and an increase in cell size. **(a)** Scanning electron micrograph (SEM) and **(d)** tangential section of control eyes. **(b)** SEM of an eye displaying a dramatic outgrowth due to an increase in ommatidia number. This phenotype is caused by the overexpression of *UAS-dinr*^{wt} with *ey-Gal4* in proliferating eye precursor cells. A tangential section of the corresponding eye **(e)** shows that normal ommatidial arrangement and architecture are retained. **(c,f)** Eyes overexpressing *UAS-dinr*^{wt} in clones under the control of the GMR enhancer, which is mainly activated in differentiating cells. Externally, such clones display enlarged ommatidial units compared with the surrounding wild-type ommatidia (c). In tangential sections (f), these clones (less pigment) contain enlarged ommatidia with enlarged photoreceptors and increased interommatidial distance. The magnification in (d–f) is the same. The genotypes are (a,d) *y w; ey-Gal4/+* (b,e) *y w; ey-Gal4/UAS-dinr*^{wt} and (c,f) *y w hsFlp; GMR>w*$^{+}$*; >Gal4/UAS-dinr*^{wt}.

378 ± 8 compared to 683 ± 8 in heterozygous control flies (Figure 1g). We did not observe any dominant size reduction with various *dinr* alleles.

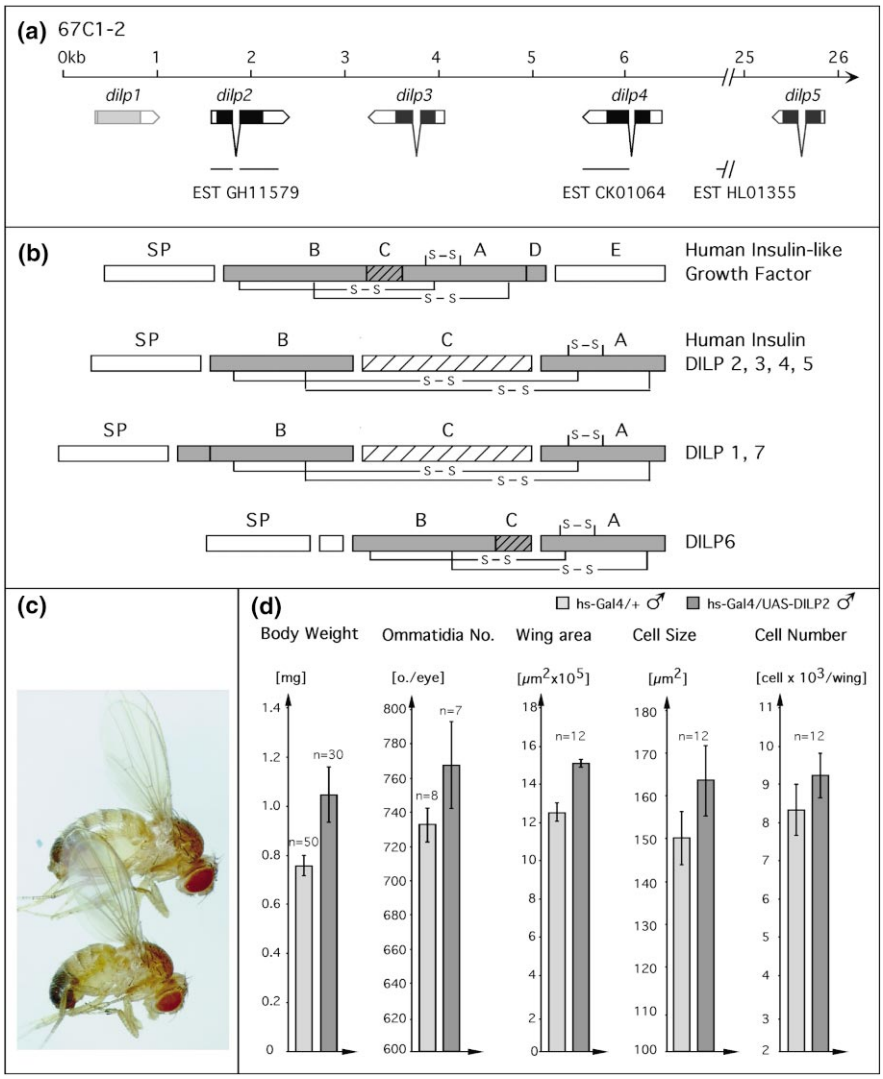
The reduced overall size could be due to DInr acting in the humoral regulation of growth or to it functioning autonomously in a cell- and tissue-specific manner. To test whether DInr affects body parts autonomously, we selectively removed DInr function in the eye imaginal disc using the ey-FLP technique [17]. The eye imaginal disc gives rise to the adult eye and the head capsule. Mosaic flies with heads largely homozygous for various *dinr* alleles displayed a dramatic reduction in eye tissue and in the head capsule, whereas the other body parts were of wild-type size (Figure 1b,e). Notably, the head size was dependent on the allele. This allowed us to arrange the alleles according to their phenotypic strength (compare Figure 1b with 1e). The strongest reduction in head size was observed with *dinr*^{339}, a putative null allele (see below), followed by *dinr*^{31}, *dinr*^{211}, *dinr*^{E19}, and *dinr*^{353}. Thus, DInr regulates head size autonomously.

Comparison of homozygous mutant tissue with heterozygous tissue in tangential sections of mosaic eyes (Figure 1c,f) revealed an estimated reduction in ommatidial size of one third for *dinr*^{E19} homozygous mutant tissue (Figure 1c) and of more than half for a candidate null allele (Figure 1f). Importantly, this growth defect does not impede proper cell fate determination, given that the normal arrangement of the photoreceptor rhabdomeres is retained (Figure 1c,f). Furthermore, the cell size reduction is cell autonomous, as can be seen at the border between homozygous mutant tissue and heterozygous tissue; within the same ommatidial unit, small homozygous cells (arrowhead in Figure 1c,f) coexist with normal-sized heterozygous cells. Although cells lacking DInr function survive and differentiate normally, they have a growth disadvantage compared to heterozygous cells. When homozygous mutant cell clones are induced during early larval life and analyzed in the imaginal discs in the third instar, clone size is greatly reduced compared to the wild-type sister clone (Figure 1d). The phenotypes of *dinr* mutant cells are strikingly similar to those of mutants in the PI(3)K/PKB pathway. Therefore, it is likely that DInr directly regulates cell growth at least in part through the PI(3)K/PKB pathway.

Identification of mutations affecting conserved amino acid residues of the kinase domain of DInr

The structure of DInr is similar to the mammalian insulin receptor (Inr) and the IGF1 receptor (IGF1R) [15]. It is a tetramer composed of two α subunits containing the putative ligand binding domains and two transmembrane β subunits containing the cytoplasmic tyrosine kinase domains. In contrast to human receptors, DInr possesses extensions at the amino and carboxy termini. The C-terminal extension contains binding sites for downstream components similar to those found in insulin receptor substrates (IRS), and has been shown to be able to signal in the absence of IRS proteins [18]. Furthermore, genetic evidence in *Drosophila* suggests that DInr can signal in the absence of Chico, the IRS1-4 homolog [7]. In order to understand the molecular basis for differences in strength of DInr phenotypes, we sequenced the cyto-

Figure 3



A new family of genes encoding putative insulin-related peptides. **(a)** Genomic organization of the *dilp* gene cluster containing *dilp1–5* mapping to 67C1-2 on the third chromosome. Genomic DNA (GenBank accession number AE003550.1) is represented by a line (top) with distances in kb and an arrow pointing to the centromere. Black boxes indicate exons of *dilps* for which mRNA expression has been detected (Table 1). A gray box indicates the predicted exon of *dilp1*, which did not show mRNA expression and therefore may be a pseudogene. Exon–intron boundaries were determined by comparing genomic DNA and expressed sequence tags (EST; bottom lines), or were predicted by the Genscan program [38]. *dilp5* is separated by one intervening gene from *dilp4*. Interestingly, each intron splits the reading frame between position 1 and 2 of a codon; this is typical for insulin-related genes [46]. **(b)** Schematic representation of the predicted structure of *Drosophila* insulin-like peptides (DILP1–7) and comparison with human IGF and insulin. Domains are denoted by letters. The spaces between domains represent predicted proteolytic cleavage [26] during maturation of the propeptide (see also supplementary figure). The active peptides (dark boxes) consist of one polypeptide chain (IGF) or two chains (DILP1–7 and human insulin). Disulfide bonds between conserved cysteines are indicated. **(c)** Proportionate increase in body size of male flies overexpressing DILP2 (top). To achieve ubiquitous expression of DILP2, we used an *hs-Gal4* driver line (Bloomington stock center). Heat shocks at 37°C were applied for 1 hr every 12 hr during development, starting at 24 hr AED. Genotypes are *y w; hs-Gal4/UAS-dilp2* (top) and *y w; hs-Gal4/+* (bottom). **(d)** DILP2 overexpression increases organismal size by increasing the cell size and cell number of individual organs. Compared to control flies, body weight and number of ommatidia are increased by 39% and 5%, respectively. The wing area is increased by 21% due to a significant increase in cell size and cell number by 9% and 11%, respectively. Values are means ± standard deviation; all values of *hs-Gal4/UAS-dilp2* males are significantly increased relative to *hs-Gal4/+* control flies (t-test, $p < 0.001$). Male flies that were 2–3 days old were weighed and analyzed. Similar results were obtained with female flies. Five independent *UAS-dilp2* transgenic lines yielded similar results (n = 16–78).

plasmic region of several *dinr* alleles [15, 16, 19]. In the cytoplasmic portion, 5 out of 22 alleles carry a point mutation. All of them map to conserved amino acid residues within the kinase domain. Two of these point mutations lead to premature stop codons and three are missense mutations (Figure 1h). In humans, most of the mutations that occur within the tyrosine kinase domain of the Inr have been shown to impair insulin-stimulated tyrosine kinase activity [20]. *dinr*^{353} (Arg1419Cys) affects an active site residue, which mediates insulin receptor kinase substrate specificity [21]. Remarkably, a human patient with severe growth retardation associated with insulin resistance, a syndrome called leprechaunism, carries an amino acid exchange at the corresponding position (Arg1092Glu) [22]. It is the only reported homozygous viable mutation in the kinase domain of the human Inr. The patient's parents were heterozygous for this substitution and had severe insulin resistance, but no growth anomalies. Similarly, heterozygosity for *dinr* alleles does not lead to growth phenotypes. These results suggest a role for the insulin receptor in growth control that has been conserved from insects to humans.

Overexpression of DInr in the eye leads to hyperproliferation and an increase in cell size

It has been proposed that a bona fide growth control gene should meet two criteria [6], namely that elimination

Table 1

Summary of *dilp* expression in embryos and larvae.

Gene	Embryo	Larva
dilp1	no signal	N.D.
dilp2	high signal in midgut, low signal in mesoderm stage 12–16	ubiquitous low signal in imaginal discs, high signal in seven cells of each brain hemisphere and in salivary glands
dilp3	no signal	high signal in seven cells of each brain hemisphere
dilp4	high signal in mesoderm stage 2–6, anterior midgut rudiment	high expression in midgut
dilp5	no signal	high signal in seven cells of each brain hemisphere, moderate signal in gut
dilp6	no signal	low signal in gut
dilp7	ubiquitous (except yolk) low signal, moderate signal in midgut	high signal in ten cells of ventral nerve cord

N.D., not determined

should result in growth retardation, whereas overexpression of the gene should promote excessive growth. To determine whether DInr has a direct growth- and proliferation-promoting effect, we overexpressed a wild-type *dinr* cDNA using the UAS/Gal4 system [23]. Expressing *UAS-dinr*^{WT} specifically in proliferating eye precursor cells using an *eyeless-Gal4* driver resulted in a dramatic outgrowth in the adult eye because of an increase in the number of ommatidia (Figure 2b). Histological sections through the overgrown eyes revealed essentially normal cell differentiation but a slight increase in the size of photoreceptor cell bodies (Figure 2e). To further explore the effect on cell size, we overexpressed DInr in clones of cells during cell differentiation. External observation of such clones showed strongly enlarged ommatidia (Figure 2c). Histological sections revealed a cell-autonomous increase in photoreceptor cell size but only a moderate disruption of the ommatidial pattern (Figure 2f). Taken together, these results indicate that DInr activity controls growth in two ways: by regulating cell proliferation and cell size. Interestingly, although overexpression of Dp110 has been shown to increase cell size, it does not increase cell division rates (data not shown) [9]. The IRS homolog Chico contains consensus binding sites for the Drk/Grb2 adaptor and thus may provide a link to the Ras/MAPK pathway. Activation of DInr may promote cell growth and cell division by activation of two signaling pathways. Indeed, MAPK activation is observed in extracts of heads overexpressing an activated form of DInr (Sean Oldham and E.H., unpublished observation).

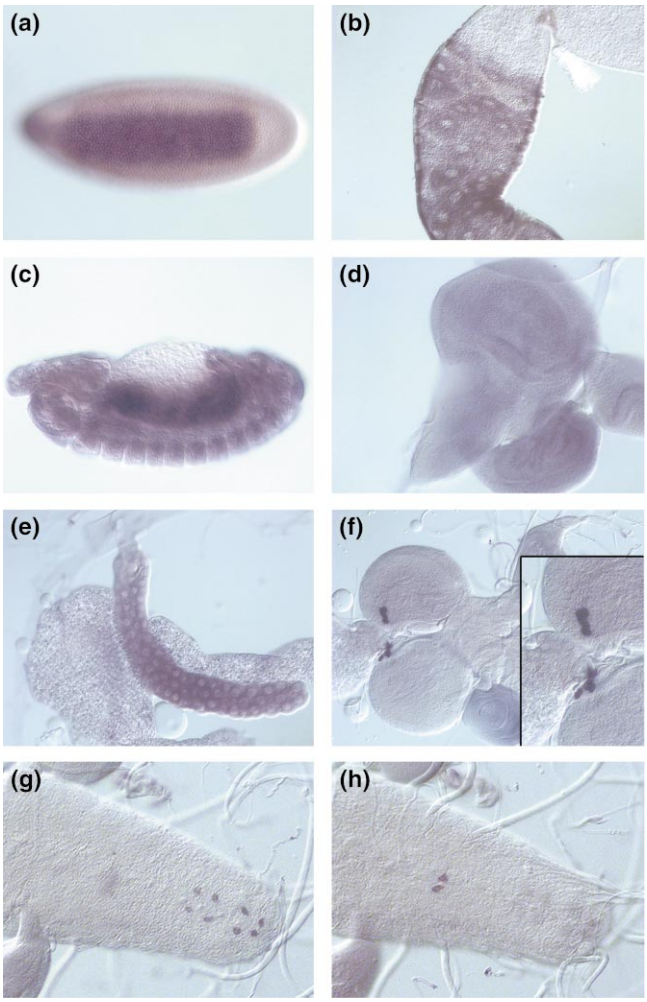
Identification of a new family of genes encoding putative insulin-related peptides

To identify extracellular ligands that regulate DInr activity during development, we searched the *Drosophila* genome [24] for genes encoding insulin-like peptides. Using the conserved spacing of four cysteines within the A chain as a signature for insulin-like peptides [25], we identified seven predicted genes matching these criteria, which we termed *dilp1–7* for *Drosophila i*nsulin-*l*ike *p*eptides. *dilp1–5* are on the third chromosome at cytological position 67C1-2, and constitute a cluster of four contiguous insulin-related genes with *dilp5* separated by one intervening gene from *dilp4* (Figure 3a). The other genes, *dilp6* and *dilp7*, are on the X chromosome at two different loci at cytological positions 2F4 and 3F2, respectively. *dilp1–7* encode putative precursor proteins of 107 to 156 amino acid residues in length that are structurally similar to preproinsulin, with a signal peptide, a B chain, a C peptide, and an A chain (Figure 3b; supplementary figure published with this paper on the internet). Consensus cleavage sites [26] between the B and A chains of all seven DILPs suggest that the active peptides consist of two separate polypeptide chains. Thus, these peptides resemble insulin rather than IGF1 or IGF2, which are single polypeptides (Figure 3b). Comparison of the amino acid sequence of the A and B chains of DILP1–7 with insulin, IGF1, and IGF2 again reveals a higher degree of identical amino acids between these peptides and insulin. DILP2 is the most closely related, with 35% identity to mature insulin (supplementary table). These structural similarities suggest that DILP1–7 are candidate ligands for DInr.

Highly regulated expression of the *Drosophila* insulin genes during development

To determine the expression pattern of the insulin-like genes, we performed in situ hybridization on embryos and larval tissues. The results are summarized in Table 1 and Figure 4. In the embryo, only *dilp2*, *4* (Figure 4a,c), and *7* are expressed at different levels in the mesoderm and midgut. It is interesting to note that the main insulin-producing organs in mammals, the Langerhans islets in the pancreas, are of endodermal origin [27]. Four of the seven genes show a remarkably specific and unique pattern of expression in larvae. *dilp2*, *3*, and *5* display high expression levels in seven cells of anteromedial localization in the brain hemispheres that may correspond to neurosecretory cells (Figure 4f). *dilp3* is exclusively transcribed in these seven cells during larval development,

Figure 4



Spatial and temporal regulation of the expression of *Drosophila* insulin-like peptides. Wild-type embryos (a,c) and third instar larval tissues (b,d–h) after in situ hybridization with *dilp* antisense probes. **(a)** *dilp4* mRNA is detected at the blastoderm stage in the presumptive mesoderm and in the anterior midgut rudiment. Shown is a ventral view. Expression in the mesoderm is still detected after gastrulation, but is reduced from stage 12 onward. **(b)** *dilp4* is reexpressed in the larval midgut. **(c)** *dilp2* shows a broad expression in the embryonic mesoderm starting at stage 12 and an especially intense expression in the midgut, which diminishes at late stage 16. A lateral view is shown. **(d)** *dilp2* is ubiquitously expressed in third larval imaginal discs and in the salivary glands **(e)**. In addition, *dilp2* mRNA is specifically transcribed in seven cells of each brain hemisphere **(f)**. The right panel is a high magnification image. Expression in these seven cells is also detected for *dilp3* and *5*. **(g)** *dilp7* is expressed in a segmental fashion in the ventral nerve cord in four pairs of ventrally located cells in the posterior-most segments and **(h)** in one pair of dorsally located cells in the abdominal segment A1 or A2. Anterior is to the left in (a,c,f,g,h). Note that sense probes of *dilps* did not detect any specific signals in all experiments except for the *dilp2* sense probe, which was uniquely detected in the same seven cells of each brain hemisphere as for the *dilp2*, *3*, and *5* antisense probes. This may be explained by the fact that *dilp3* is located adjacent to and in the opposite orientation of *dilp2* in the genome. Therefore, it is possible that during *dilp3* transcription, the polymerase reads through into the *dilp2* region and, thus, the *dilp2* sense probe could hybridize to the *dilp2* region of the *dilp3* transcript. Consistently, *dilp3* mRNA is also uniquely detected in those seven cells of the brain during development.

whereas *dilp2* (Figure 4c,d,e) and *dilp5* show additional expression domains. *dilp7* mRNA detection is restricted to the ventral nerve cord in a segmental fashion, in four pairs of ventrally located cells in the most posterior abdominal segments and in one pair of dorsally located cells in A1 or A2 (Figure 4g,h). Interestingly, neither of the *dilps* shows detectable levels of expression in the larval fat body.

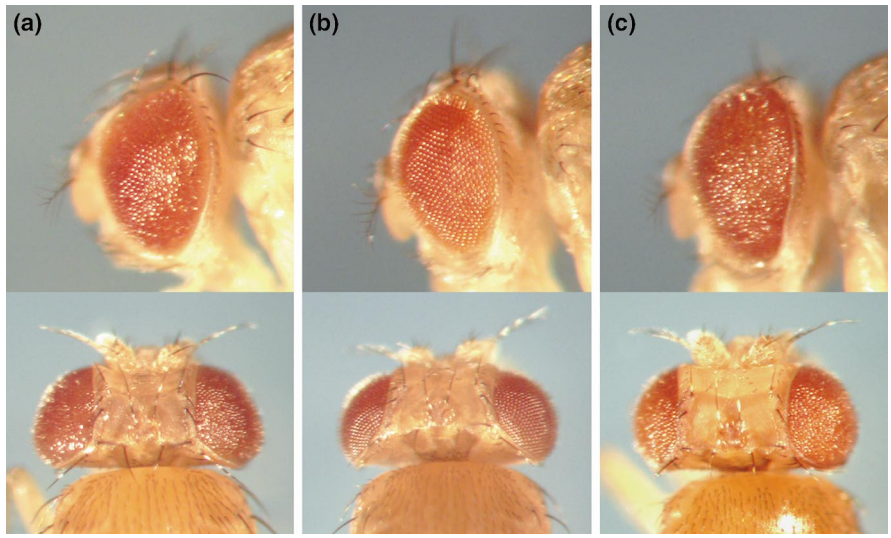
Expression of insulin-related genes in neurosecretory cells has been identified in other invertebrates, such as the insects *Bombyx mori* and *Locusta migratoria* and in the mollusc *Lymnaea stagnalis* [28–30]. In *Bombyx mori*, the neurosecretory cells in the brain are connected to the corpora cardiaca, a secretory gland from which release of insulin-like hormones is triggered by nutrient levels (possibly carbohydrate levels) [31]. We speculate that DILP-expressing neurosecretory cells are connected to the ring gland (the compound endocrine gland of *Drosophila*), which includes the cells of the corpora cardiaca. Release of DILPs from the ring gland may also be under nutritional control. The complex expression pattern of the DILPs, however, suggests a combination of neurosecretory and autocrine/paracrine control mechanisms of cell growth and division during larval development. Mutations in individual *dilp* genes or targeted ablation of specific DILP-expressing cells may help resolve the functions of the *Drosophila* insulins.

DILP2 overexpression increases organismal size by increasing cell size and cell number of individual organs

To gain insight into the function of the DILPs, we overexpressed one insulin-like peptide. For this purpose, we chose DILP2 because it is the closest homolog of human insulin (see supplementary table) and because it is the only DILP with broad expression in imaginal discs (Figure 4d). If DILP2 is a limiting ligand of DInr, we expect that overexpression of DILP2 should promote growth. Indeed, repeated induction of ubiquitous expression of DILP2 during development by means of the UAS/Gal4 system gives rise to bigger flies (39% increase in body weight; Figure 3c,d). Analysis of the eyes of such flies revealed an increase in the number of ommatidia (from 733 $\pm$ 10 to 767 $\pm$ 25 in male flies). Furthermore, quantitative analysis of the wing blade showed an increase in both cell size (by 9%) and cell number (by 11%). These results suggest a role for DILP2 in controlling organismal size by augmenting both cell number and cell size of different organs.

In humans, the in vivo role of insulin as a growth factor is inferred from clinical syndromes, in which excessive

Figure 5



Dominant suppression of a DInr-mediated big eye phenotype by a deficiency uncovering *dilp1–5*. **(a)** Overexpression of *UAS-dinr*^{wt} with the *GMR-Gal4* driver line in differentiating eye cells leads to bulging and rougher eyes. **(b)** *Df(3L)AC1* dominantly suppresses the big eye phenotype caused by the overexpression of DInr. **(c)** Introducing one copy of *UAS-dilp2* efficiently counteracts the suppressive effect of *Df(3L)AC1*. The crosses were performed at 18°C. Flies are of the following genotypes: (a) *y w; GMR-Gal4, UAS-dinr*^{wt}/+, (b) *y w; GMR-Gal4, UAS-dinr*^{wt}/+; *Df(3L)AC1*/+, and (c) *y w; GMR-Gal4, UAS-dinr*^{wt}/*UAS-dilp2; Df(3L)AC1*/+.

insulin secretion results in excessive growth and where a severe deficiency of insulin secretion is associated with poor intrauterine and postnatal growth [32]. For instance, neonates born to women with diabetes in pregnancy or born with Beckwith-Wiedemann syndrome or Nesidioblastosis are macrosomic. In all cases, the growth anomaly is associated with hyperinsulinemia during embryonic development [32–35]. Our demonstration in transgenic flies that overexpression of an insulin-like peptide during development can increase animal size provides further evidence for an evolutionarily conserved role of the insulin pathway in growth control.

DILP2 genetically interacts with DInr

The complementarity between the loss-of-function phenotype of *dinr* and the DILP2 overexpression phenotype (increase in size) suggests that DILP2 may be one of the ligands for DInr. We found that a deficiency (*Df(3L)AC1*) uncovering *dilp1–5* dominantly suppressed the big and rough eye phenotype caused by targeted overexpression of DInr in differentiating eye cells (Figure 5a,b). To test whether the observed dominant suppression was caused by hemizygosity for *dilp2*, we selectively increased the *dilp2* gene dosage by crossing in the *UAS-dilp2* transgene. A single copy of *UAS-dilp2* was sufficient to revert the suppression by *Df(3L)AC1* (Figure 5c), strongly suggesting that *dilp2* is rate limiting for the DInr overexpression phenotype. An analysis of individually mutated *dilp* genes will be required to determine the contribution of the other *dilps* of the cluster (*dilp1* and *3–5*) to the suppressive effect of *Df(3L)AC1*.

To examine whether DInr is limiting for the DILP2 overexpression phenotype, we lowered DInr activity in a DILP2-overexpressing background. Indeed, introducing one mutant copy of *dinr* (*dinr*^{304}) dominantly reduces the increased body weight, cell size, and cell number caused by ubiquitous DILP2 overexpression, indicating a strong genetic interaction between *dinr* and *dilp2* (data not shown). Persistent expression of DILP2 under the control of an *actin* promoter (*Act5C-Gal4*) caused embryonic lethality. This lethality is dependent on normal levels of DInr, as expression of DILP2 in the presence of strongly reduced levels of DInr (see Materials and methods for genotypes) generated viable adults that were small and developmentally delayed. These results are consistent with DInr mediating the effects of DILP2. Furthermore, given that a viable heteroallelic combination of *dPKB* alleles is also able to suppress the embryonic lethal phenotype of DILP2 overexpression, we postulate that the action of DILP2 by DInr is transduced at least in part through the Chico/PI(3)K/dPKB pathway.

Conclusions

In humans, syndromes with mutations in the insulin receptor or with excessive insulin secretion lead to growth abnormalities. This study shows in vivo that altering expression levels of a *Drosophila* insulin-like gene and varying the activity of the *Drosophila* insulin receptor changes the size and number of cells in organs, thereby regulating organismal size. It seems, therefore, that the insulin receptor pathway has been conserved during evolution for a role in growth control from insects to humans. Given the highly tissue-specific expression of the *dilps* in the central nervous system and a broad expression in precursor tissues of adult organs, we propose a nutritionally regulated

mechanism whereby *Drosophila* insulin-like peptides coordinate growth in a neurosecretory and local fashion.

Materials and methods

Generation of mosaic flies by mitotic recombination

The *dinr* alleles (*E19*, *31*, *304*, *211*, *339*, and *353*) were individually recombined onto the FRT 82B chromosome [36]. Mosaic heads were generated with the ey-FLP/FRT recombination system [17], which drives the FLP recombinase under the control of the *eyeless* enhancer, thereby inducing mitotic recombination in eye progenitor cells of embryos that are heterozygous for different *dinr* alleles. A recessive cell lethal mutation on the homologous chromosome prevents the growth of the sister clone. Thus, the *dinr* homozygous mutant clone contributes the majority of cells in the eye and the head capsule. Such flies have heads that are largely homozygous mutant while the rest of the body is heterozygous. To establish an allelic series, we determined the growth defect of each mosaic head ($n = 10$) by measuring the ratio between head and thorax widths and by analyzing the cell size defect in histological sections. The head to thorax ratio of mosaic heads (and control flies) are, in the order of severity, $dinr^{339}$: 0.68 (1.23), $dinr^{31}$: 0.74 (1.20), $dinr^{211}$: 0.79 (1.22), $dinr^{E19}$: 0.81 (1.16), and $dinr^{353}$: 0.82 (1.19). The differences in head size compared to controls and between the alleles analyzed were significant ($p < 0.0001$ and $p < 0.005$).

To generate clones of homozygous *dinr* mutant cells in imaginal discs, *y w hsFLP/y w; FRT82B* $dinr^{304}$*/FRT82B P(arm-lacZ,* w^+*)* larvae were heat shocked 24–48 hr AED for 0.5 hr at 34°C to induce expression of the FLP recombinase. Larvae were dissected at the late third instar stage. Discs were fixed, permeabilized, and stained with mouse anti-β-gal (1/1000) and FITC-conjugated secondary antibodies (1/200).

Measurements of cell number, cell size, and weight

Each cell in the wing blade gives rise to a single wing hair. Thus, the cell density was assessed by counting the number of wing hairs on the dorsal wing surface in a 10,000 μm^2 area just posterior to the posterior cross vein. The reciprocal value of the cell density is the cell area. The approximate number of cells in the whole wing was calculated by multiplying the cell density by the wing area excluding the alula and the costal cell. NIH Image 1.60 was used to measure the wing area. Individual male and female flies were weighed with a precision scale (Mettler ME30, range 0.001–10 mg). Flies homozygous for $dinr^{E19}$ were obtained after removing linked lethal mutations by recombination.

Molecular analysis of dinr alleles

The following *dinr* alleles were sequenced: *E16*, *E19*, *E21*, *31*, *EC34*, *35*, *76*, *87*, *117*, *211*, *242*, *262*, *273*, *277*, *304*, *306*, *310*, *313*, *322*, *327*, *339*, and *353* [15, 16, 19]. Genomic DNA was extracted from heterozygous flies balanced over *TM3 Sb*. The region coding for the cytoplasmic portion was amplified by PCR (primer sequences are available on request), sequenced, and analyzed with Sequencher™ software and compared to the published sequence [15].

Generation of transgenic flies

The *dinr* cDNA was subcloned as an EcoRI fragment into the pUAST vector to generate transgenic flies by means of P element–mediated germline transformation. To achieve expression of *UAS-*$dinr^{wt}$ in proliferating eye precursor cells, the *ey-Gal4* driver line was used [37]. To achieve expression of *UAS-*$dinr^{wt}$ in clones of eye precursor cells undergoing a final round of division and subsequent differentiation, we induced Gal4 expression under the control of the GMR enhancer by heat shocking wandering larvae for 15 min at 34°C containing a heat shock–inducible FLP recombinase and a flp-out transgene (*GMR>*w^+*,STOP>Gal4*). A full-length cDNA clone, EST GH11579 (obtained from Research Genetics), was used to subclone *dilp2* as a 0.65 kb EcoRI/XhoI fragment into pUAST.

Genome search for insulin-like genes, gene structure, and protein structure analysis

We searched for insulin-like genes in the *Drosophila* nucleotide database with the human A chain, which yielded a full-length *dilp2* cDNA clone, GH11579. We then used the predicted *Drosophila* A chains to identify a total of seven insulin-like genes. Searches with A chains were performed with the TBLASTN program (National Center for Biotechnology Information). Gene prediction and conceptual translation was performed with Genscan [38], and signal peptides were predicted using the SignalP program [39]. Cleavage sites were predicted at either specific single or pairs of basic residues of the general formula (R/K)-Xn-(R/K), where cleavage preference decreases with $n = 0$, 2, 4, or 6 [26].

The Berkeley *Drosophila* Genome Project (BDGP) [24] has identified four genes as being insulin-like (corresponding to *dilp1–4*). *dilp5* has not been predicted by BDGP. *dilp5* has its predicted translational start site at nucleotide position 237,598 and stop codon at 237,990 within the contig (nucleotide positions refer to GenBank accession number AE003550.1). We found that the *dilp3* gene consists of two exons and has its translational start at 259,432 and stop at 259,854. Corresponding gene numbers in the genome annotation database of *Drosophila* (GadFly) for *dilp1–4* and *dilp6* and 7 are: CG14173, CG8167, CG14167, CG6736, CG14049, and CG13317.

In situ hybridization

In situ hybridization to larval tissues and embryos was performed essentially as described [40, 41]. The genomic region including *dilp* genes was PCR amplified and cloned into the pCRII-Topo vector (Invitrogen) except for *dilp2* and *dilp4,* which we obtained as an EST clone (LD06542 and GH11579) from Research Genetics. In vitro transcription was done using the DIG RNA labeling kit (Roche). Digoxigenin (DIG)–labeled RNA sense probes were transcribed by SP6 polymerase, and antisense probes by T7 polymerase. Hydrolysis time was shortened to 5 min to prevent cross-hybridization.

Genetic interaction analysis

Df(3L)AC1 (breakpoints 67A2; 67D13, Flybase) removes *dilp1–5*, as *dshc* (67B3) [42], which lies distal to the *dilp* cluster (67C1-2), and *Gap1* (67C2-3) [43], which lies proximal to the *dilp* cluster, are both uncovered by this deficiency. Targeted overexpression of *UAS-*$dinr^{wt}$ in differentiating eye cells by means of *GMR-Gal4* [44] leads to bulging rough eyes (Figure 5a), a phenotype that is dominantly suppressed by *Df(3L)AC1* (Figure 5b). Introducing one copy of *UAS-dilp2* restores the big eye phenotype (Figure 5c). This cannot simply be an additive effect because in a wild-type background, overexpression of *USA-dilp2* under the control of *GMR-Gal4* is phenotypically neutral.

The lethality resulting from overexpression of *UAS-dilp2* with *Act5C-Gal4* (Bloomington stock center) was rescued in the following genotypic backgrounds, in order of decreasing viability: (1) *y w; Act5C-Gal4/UAS-dilp2;* $dinr^{05545}$*/*$dinr^{211}$, (2) *y w; Act5C-Gal4/UAS-dilp2;* $dinr^{05545}$*/*$dinr^{E19}$, (3) *y w; Act5C-Gal4/UAS-dilp2;* $dPKB^{1}$*/* $dPKB^{3}$, (4) *y w; Act5C-Gal4/UAS-dilp2;* $dinr^{E19}$*/*$dinr^{211}$, (5) *y w; Act5C-Gal4/UAS-dilp2;* $dinr^{E19}$*/* $dinr^{E19}$, and (6) *y w; Act5C-Gal4/UAS-dilp2;* $dinr^{304}$*/+*.

Supplementary material

Supplementary material including a figure showing an alignment of the predicted amino acid sequences of *Drosophila* insulin-like peptides with human preproinsulin, and a table comparing the predicted mature DILP1–7 with human insulin and IGFs are available at http://www.current-biology.com/supmat/supmatin.htm.

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References

1. Stern DL, Emlen DJ: **The developmental basis for allometry in insects.** *Development* 1999, **126:**1091-1101.
2. Britton JS, Edgar BA: **Environmental control of the cell cycle in *Drosophila*: nutrition activates mitotic and endoreplicative cells by distinct mechanisms.** *Development* 1998, **125:**2149-2158.
3. Partridge L, Barrie B, Fowler K, French V: **Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature.** *Evolution* 1994, **48:**1269-1276.
4. Robertson FW: **Studies in quantitative inheritance. XII. Cell size and number in relation to genetic and environmental variation of body size in *Drosophila*.** *Genetics* 1959, **44:**869-896.
5. Conlon I, Raff M: **Size control in animal development.** *Cell* 1999, **96:**235-244.
6. Efstratiadis A: **Genetics of mouse growth.** *Int J Dev Biol* 1998, **42:**955-976.
7. Bohni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andruss BF, *et al.*: **Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4.** *Cell* 1999, **97:**865-875.
8. Leevers SJ, Weinkove D, MacDougall LK, Hafen E, Waterfield MD: **The *Drosophila* phosphoinositide 3-kinase Dp110 promotes cell growth.** *EMBO J* 1996, **15:**6584-6594.
9. Weinkove D, Neufeld TP, Twardzik T, Waterfield MD, Leevers SJ: **Regulation of imaginal disc cell size, cell number and organ size by *Drosophila* class I(A) phosphoinositide 3-kinase and its adaptor.** *Curr Biol* 1999, **9:**1019-1029.
10. Goberdhan DC, Paricio N, Goodman EC, Mlodzik M, Wilson C: ***Drosophila* tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway.** *Genes Dev* 1999, **13:**3244-3258.
11. Huang H, Potter CJ, Tao W, Li DM, Brogiolo W, Hafen E, *et al.*: **PTEN affects cell size, cell proliferation and apoptosis during *Drosophila* eye development.** *Development* 1999, **126:**5365-5372.
12. Gao X, Neufeld TP, Pan D: ***Drosophila* PTEN regulates cell growth and proliferation through PI3K- dependent and -independent pathways.** *Dev Biol* 2000, **221:**404-418.
13. Verdu J, Buratovich MA, Wilder EL, Birnbaum MJ: **Cell-autonomous regulation of cell and organ growth in *Drosophila* by Akt/PKB.** *Nat Cell Biol* 1999, **1:**500-506.
14. Montagne J, Stewart MJ, Stocker H, Hafen E, Kozma SC, Thomas G: ***Drosophila* S6 kinase: a regulator of cell size.** *Science* 1999, **285:**2126-2129.
15. Fernandez R, Tabarini D, Azpiazu N, Frasch M, Schlessinger J: **The *Drosophila* insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential.** *EMBO J* 1995, **14:**3373-3384.
16. Chen C, Jack J, Garofalo RS: **The *Drosophila* insulin receptor is required for normal growth.** *Endocrinology* 1996, **137:**846-856.
17. Newsome TP, Asling B, Dickson BJ: **Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics.** *Development* 2000, **127:**851-860.
18. Yenush L, Fernandez R, Myers MG Jr, Grammer TC, Sun XJ, Blenis J, *et al.*: **The *Drosophila* insulin receptor activates multiple signaling pathways but requires insulin receptor substrate proteins for DNA synthesis.** *Mol Cell Biol* 1996, **16:**2509-2517.
19. Azpiazu N, Frasch M: **Tinman and bagpipe: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*.** *Genes Dev* 1993, **7:**1324-1340.
20. Baynes KC, Whitehead J, Krook A, O'Rahilly S: **Molecular mechanisms of inherited insulin resistance.** *QJM* 1997, **90:**557-562.
21. Hubbard SR, Wei L, Ellis L, Hendrickson WA: **Crystal structure of the tyrosine kinase domain of the human insulin receptor.** *Nature* 1994, **372:**746-754.
22. Takahashi Y, Kadowaki H, Momomura K, Fukushima Y, Orban T, Okai T, *et al.*: **A homozygous kinase-defective mutation in the insulin receptor gene in a patient with leprechaunism.** *Diabetologia* 1997, **40:**412-420.
23. Brand AH, Perrimon N: **Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.** *Development* 1993, **118:**401-415.
24. Adams MD, *et al.*: **The genome sequence of *Drosophila melanogaster*.** *Science* 2000, **287:**2185-2195.
25. Blundell TL, Humbel RE: **Hormone families: pancreatic hormones and homologous growth factors.** *Nature* 1980, **287:**781-787.
26. Seidah NG, Chretien M: **Eukaryotic protein processing: endoproteolysis of precursor proteins.** *Curr Opin Biotechnol* 1997, **8:**602-607.
27. Madsen OD, Jensen J, Blume N, Petersen HV, Lund K, Karlsen C, *et al.*: **Pancreatic development and maturation of the islet B cell: studies of pluripotent islet cultures**. *Eur J Biochem* 1996, **242:**435-445.
28. Kawakami A, Iwami M, Nagasawa H, Suzuki A, Ishizaki H: **Structure and organization of four clustered genes that encode bombyxin, an insulin-related brain secretory peptide of the silkmoth *Bombyx mori*.** *Proc Natl Acad Sci USA* 1989, **86:**6843-6847.
29. Hetru C, Li KW, Bulet P, Lagueux M, Hoffmann JA: **Isolation and structural characterization of an insulin-related molecule, a predominant neuropeptide from *Locusta migratoria*.** *Eur J Biochem* 1991, **201:**495-499.
30. Smit AB, Vreugdenhil E, Ebberink RH, Geraerts WP, Klootwijk J, Joosse J: **Growth-controlling molluscan neurons produce the precursor of an insulin-related peptide.** *Nature* 1988, **331:**535-538.
31. Masumura M, Satake S, Saegusa H, Mizoguchi A: **Glucose stimulates the release of bombyxin, an insulin-related peptide of the silkworm *Bombyx mori*.** *Gen Comp Endocrinol* 2000, **118:**393-399.
32. Menon RK, Sperling MA: **Insulin as a growth factor.** *Endocrinol Metab Clin North Am* 1996, **25:**633-647.
33. Breschi MC, Seghieri G, Bartolomei G, Gironi A, Baldi S, Ferrannini E: **Relation of birthweight to maternal plasma glucose and insulin concentrations during normal pregnancy.** *Diabetologia* 1993, **36:**1315-1321.
34. Elliott M, Bayly R, Cole T, Temple IK, Maher ER: **Clinical features and natural history of Beckwith-Wiedemann syndrome: presentation of 74 new cases.** *Clin Genet* 1994, **46:**168-174.
35. Milner RD: **Nesidioblastosis unravelled.** *Arch Dis Child* 1996, **74:**369-372.
36. Xu T, Rubin GM: **Analysis of genetic mosaics in developing and adult *Drosophila* tissues.** *Development* 1993, **117:**1223-1237.
37. Halder G, Callaerts P, Flister S, Walldorf U, Kloter U, Gehring WJ: **Eyeless initiates the expression of both sine oculis and eyes absent during *Drosophila* compound eye development.** *Development* 1998, **125:**2181-2191.
38. Burge C, Karlin S: **Prediction of complete gene structures in human genomic DNA.** *J Mol Biol* 1997, **268:**78-94.
39. Nielsen H, Engelbrecht J, Brunak S, von Heijne G: **Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites.** *Protein Eng* 1997, **10:**1-6.
40. O'Neill JW, Bier E: **Double-label in situ hybridization using biotin and digoxigenin-tagged RNA probes.** *Biotechniques* 1994, **17:**870.
41. Lehmann R, Tautz D: ***In situ* hybridization to RNA.** In *Drosophila melanogaster*. *Practical Use in Cell and Molecular Biology*. Edited by Goldstein LSB and Fyrberg EA. San Diego: Academic Press; 1994:575-598.
42. Luschnig S, Krauss J, Bohmann K, Desjeux I, Nusslein-Volhard C: **The *Drosophila* SHC adaptor protein is required for signaling by a subset of receptor tyrosine kinases.** *Mol Cell* 2000, **5:**231-241.
43. Gaul U, Mardon G, Rubin GM: **A putative Ras GTPase activating protein acts as a negative regulator of signaling by the Sevenless receptor tyrosine kinase.** *Cell* 1992, **68:**1007-1019.
44. Hay BA, Wolff T, Rubin GM: **Expression of baculovirus P35 prevents cell death in *Drosophila*.** *Development* 1994, **120:**2121-2129.
45. Ebina Y, Ellis L, Jarnagin K, Edery M, Graf L, Clauser E, *et al.*: **The human insulin receptor cDNA: the structural basis for hormone-activated transmembrane signalling.** *Cell* 1985, **40:**747-758.
46. Duret L, Guex N, Peitsch MC, Bairoch A: **New insulin-like proteins with atypical disulfide bond pattern characterized in *Caenorhabditis elegans* by comparative sequence analysis and homology modeling.** *Genome Res* 1998, **8:**348-353.

PDK1 regulates growth through Akt and S6K in *Drosophila*

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PDK1 regulates growth through Akt and S6K in *Drosophila*

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The insulin/insulin-like growth factor-1 signaling pathway promotes growth in invertebrates and vertebrates by increasing the levels of phosphatidylinositol 3,4,5-triphosphate through the activation of p110 phosphatidylinositol 3-kinase. Two key effectors of this pathway are the phosphoinositide-dependent protein kinase 1 (PDK1) and Akt/PKB. Although genetic analysis in *Caenorhabditis elegans* has implicated Akt as the only relevant PDK1 substrate, cell culture studies have suggested that PDK1 has additional targets. Here we show that, in *Drosophila,* dPDK1 controls cellular and organism growth by activating dAkt and S6 kinase, dS6K. Furthermore, *dPDK1* genetically interacts with *dRSK* but not with *dPKN*, encoding two substrates of PDK1 *in vitro*. Thus, the results suggest that dPDK1 is required for dRSK but not dPKN activation and that it regulates insulin-mediated growth through two main effector branches, dAkt and dS6K.

Genetic studies in *Caenorhabditis elegans* and *Drosophila,* and biochemical analyses in vertebrate cell culture systems have led to the identification of key components of the insulin signal transduction pathway, including members of the phosphatidylinositol 3-kinase [PI(3)K] signaling pathway; the protein kinases PDK1, Akt, GSK3, and S6K and the 3-phosphatidylinositide phosphatase PTEN, which antagonizes the effects of PI(3)K by converting phosphatidylinositol 3,4,5-triphosphate (PIP_3) to phosphatidylinositol 4,5-biphosphate. In addition, studies *in vitro* and in vertebrate cell culture systems have implicated phosphoinositide-dependent protein kinase 1 (PDK1) as the critical regulator of T-loop phosphorylation in many members of the AGC family of kinases, which include Akt (1–6), S6K (7, 8), RSK (9, 10), PKN (11), and all isoforms of protein kinase C (12–15). PDK1 possesses two functional domains, a serine/threonine kinase domain located amino-terminally and a Pleckstrin-homology domain with a high affinity to PIP_3. Owing to this high affinity to PIP_3, PDK1 is located at the membrane even in resting cells and controls activity of its target kinases at the plasma membrane (4, 16). Consistent with PDK1 being a direct effector of Akt, S6K, and RSK, activation of all three kinases is blocked in $PDK1^{-/-}$-deficient embryonic stem cells (17). These findings imply that *in vivo* PDK1 has multiple targets and acts as a downstream branch point for PI(3)K signaling. However, despite these observations, genetic analyses in *C. elegans* and a recent study in *Drosophila* have implicated Akt as the only relevant target for PDK1 function (18, 19). In contrast, the detailed genetic analysis of dPDK1 function in *Drosophila* presented here indicates that PDK1 functions as a central regulator of cell growth by regulating two effector pathways controlled by the AGC kinases Akt and S6K, respectively.

Methods

Ethyl Methanesulfonate (EMS) Mutagenesis and Analysis of Mutants. To generate mutations in *dPDK1*, *y w*; *EP(3)0837/TM2* y^+ males were treated with EMS according to Lewis and Bacher (20) and mated to *y w*; *GMR-Gal4 UAS-dAkt/CyO*; *MKRS/TM2* females. A total number of 2,300 F_1 progeny of the genotype *y w*; *GMR-Gal4 UAS-dAkt/+*; *EP(3)0837/MKRS* or *TM2* was screened for a suppression of the big eye phenotype shown in Fig. 2*d*. Primary positives were retested, and stocks were established by balancing the potential *dPDK1* alleles with the *TM6B* y^+ balancer.

Genomic DNA was extracted from heterozygous flies, and coding exons of *dPDK1* were amplified by PCR. The PCR products were sequenced and analyzed with SEQUENCHER software for the appearance of double peaks in the sequence chromatogram, and compared with the published sequence (21). The nucleotide changes are: $dPDK1^3$ (GGT→AGT), $dPDK1^4$ (CCG→CTG), $dPDK1^5$ (CAG→TAG).

Clonal Analysis. Clonal analysis of *dPDK1* loss-of-function alleles was performed by using the *Flp/FRT* and the *ey-Flp* systems as described (22, 23). To generate marked clones that express either *EP(3)0837* controlled *dPDK1* and/or *UAS-dAkt* in eye disk cells during the last cell cycle and subsequent differentiation, 24- to 48-h-old larvae containing a heat-shock-inducible *Flp* recombinase, a Flp-out transgene (*GMR>FRT* w^+ STOP *FRT>Gal4*), the *EP(3)0837* element, and/or a *UAS-dAkt* construct were subjected to a heat shock for 1 h at 37°C. This procedure induces recombination between the *FRT* sites of *GMR>FRT* w^+ STOP *FRT>Gal4* and removes the w^+ STOP cassette in clones, thus allowing expression of *dPDK1* and *dAkt* under the control of *GMR-Gal4*. Histological sections of the eyes were performed as described (24).

Plasmids and Germ-Line Transformation. To generate *UAS-dPDK1*, the ORF coding for dPDK1 was amplified from the full-length cDNA clone LD16509 (obtained from Research Genetics, Huntsville, AL) by PCR by using the primers 5′-GGAATTCATGGCCAAGGAGAAAGCATC-3′ (ofr77) and 5′-GCTCTAGACGTTTACTTAGACGCCGTC-3′ (ofr80), which introduced *Eco*RI and *Xba*I sites at the 5′ and 3′ ends, respectively. The PCR product was ligated into the pUAST *Drosophila* transformation vector (25), and the resulting plasmid *UAS-dPDK1* was used for transformation.

To generate *UAS-PDK1*^{A467V} the point mutation C→T at *dPDK1* nucleotide position 2,032 was introduced with a QuickChange Site-Directed Mutagenesis Kit from Stratagene. For PCR we used the primers 5′-GTTTATCAGATGATCGTCGGCCTACCGCCATTC-3′ and 5′-GAATGGCGGTAGGCCGACGATCATCTGATAAAC-3′ and the pBluescript SK(−) plasmid containing the cDNA clone LD16509 as a template. The resulting plasmid was used as a template for PCR with primers ofr77 and ofr80. The PCR product was digested with *Eco*RI and *Xba*I and cloned into the pUAST vector. The resulting plasmid *UAS-PDK1*^{A467V} was used for transformation.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: EP, enhancer–promoter; PI(3)K, phosphatidylinositol 3-kinase; PDK1, phosphoinositide-dependent protein kinase 1; PIP_3, phosphatidylinositol 3,4,5-triphosphate; EMS, ethyl methanesulfonate.

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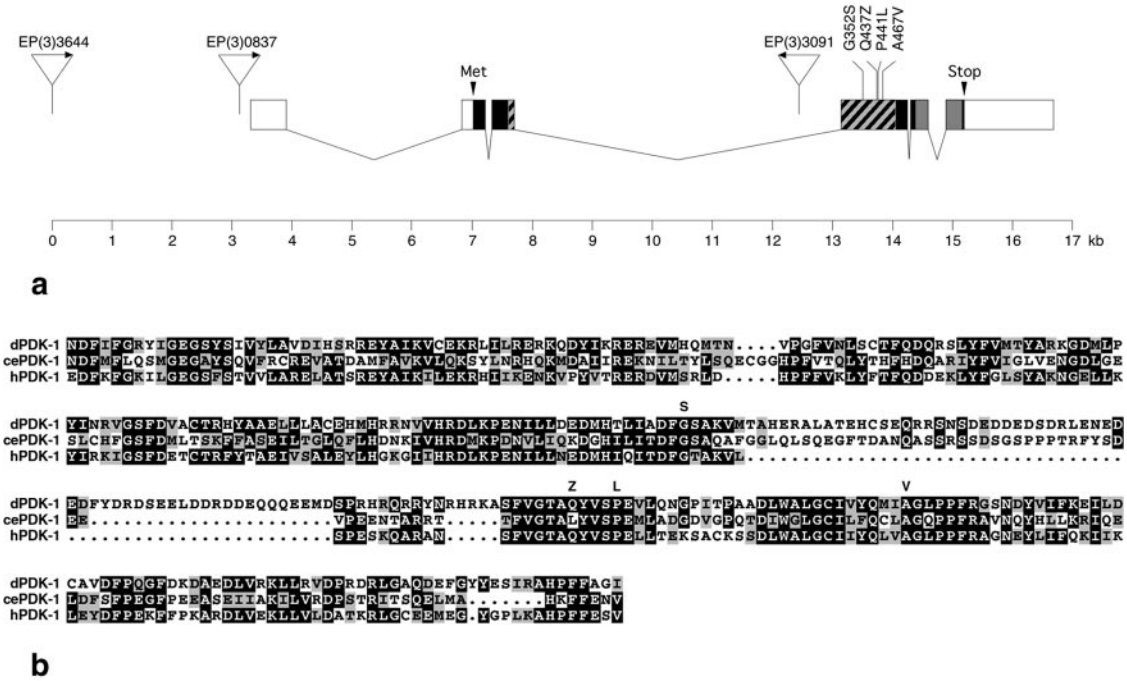

Fig. 1. Gain- and loss-of-function mutations in the *dPDK1* locus. (*a*) Genomic structure of the *dPDK1* locus. One of several reported transcripts (42) represented by the expressed sequence tag (EST) cDNA LD16509 is shown. Boxes represent exons. Dark boxes indicate the ORF; hatched and gray boxes represent the kinase and Pleckstrin-homology domains, respectively. EP insertions *EP(3)0837*, *EP(3)3091*, and *EP(3)3644* are shown as triangles, and the direction of transcription from the *UAS*-controlled promoter is marked by arrows. *EP(3)3644* inserted 7,081 and *EP(3)0837* 3,875 nt upstream of the putative start codon (Met). *EP(3)3091* inserted 710 bp upstream of the 5′ end of exon 3. The three EMS-induced loss-of-function mutations and the activating mutation A467V are shown above exon 4. (*b*) Kinase domain alignment of *Drosophila*, *C. elegans*, and human PDK1. Dark and gray boxes indicate amino acid identity and similarity, respectively. Amino acid changes in the $dPDK1^{3-5}$ and $dPDK1^{A467V}$ mutants are shown above the dPDK1 sequence. Note that the amino acid substitutions in dPDK1[3] (G352S) and dPDK1[4] (P441L) are in highly conserved amino acid residues.

To generate *UAS-dPKN* and *UAS-dRSK* we performed PCR by using as a template double-stranded cDNA derived from 0- to 24-h-old *Drosophila* embryos (kindly provided by K. Nairz, Universität Zürich, Zürich, Switzerland) with the following primer pairs: 5′-CGGCGAATTAACGAGAAACC-3′ and 5′-GGCCCGTTAGTAAATCCTTG-3′ for *dPKN* and 5′-AACAAAGGAACCGCTAGGAG-3′ and 5′-AAGTAGTCGGACTATCTGCC-3′ for *dRSK*. The PCR products were cloned by using the pCRII-TOPOTA vector system (Invitrogen). Subsequently, the *dPKN* and *dRSK* cDNAs were cut out with Asp-718 and *Not*I and ligated into the pUAST vector. The resulting plasmids *UAS-dPKN* and *UAS-dRSK* were used for transformation.

P element-mediated germ-line transformation was performed as described (26). The constructs were injected into *y w* embryos. Several independent transformant lines were established for all constructs.

Phenotypic Analysis. Unless indicated otherwise, all phenotypic analyses were done in females. Measurements of cell number, cell size, and body weight were performed as described (27). National Institutes of Health IMAGE 1.61 was used to quantify the size of ommatidia and rhabdomeres by measuring the corresponding area.

***Drosophila* Strains.** *EP(3)0837*, *EP(3)3091*, and *EP(3)3644* flies were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN). Genomic sequences flanking the 3′ end of the enhancer–promoter (EP) elements was isolated by plasmid rescue (28), sequenced, and analyzed with use of the Berkeley *Drosophila* Genome Project database (Berkeley, CA). The *Gal4* driver *GMR-Gal4* was a gift of M. Freeman (MRC Laboratory of Molecular Biology, Cambridge, U.K.), *ap-Gal4* was described in ref. 29, and *arm-Gal4* was obtained from the Bloomington *Drosophila* Stock Center. The following alleles were used for genetic interaction studies: $DPTEN^{dj189}$ [a putative null mutation caused by the insertion of an F element in a coding exon which disrupts the *dPTEN* ORF after amino acid 89 (30)], $dPTEN^{c494}$ [encoding a strong hypomorph of *dPTEN* caused by an EMS-induced amino acid exchange (G135E) in the active-site motif of the catalytic domain required for phosphatase activity (31)], $dS6K^{l-1}$ [a putative null mutation generated by imprecise excision of a P element insertion in the *dS6K* gene, which removed part of the first exon, including a portion of the catalytic domain (32)], and $dAkt^{1}$ [encoding a kinase dead version of dAkt carrying a single amino acid substitution (F327I) in the DFG motif in kinase domain VII (33)]. *ap-Gal4 UAS-dS6K* flies are described in ref. 32. The construction of *UAS-dAkt* flies will be described elsewhere. For overexpression studies in which the EMS-induced *dPDK1* alleles $dPDK1^{4}$ and $dPDK1^{5}$ were used, we induced the jump-out of the EP element *EP(3)0837* from fly stocks $dPDK1^{4}$ and $dPDK1^{5}$ to avoid overexpression of mutant dPDK1 proteins in a background where *Gal4* is expressed. P element mobilization was achieved by standard genetic techniques.

Results and Discussion

To analyze the function of dPDK1 in *Drosophila*, we aimed to generate both gain- and loss-of-function alleles of the kinase. *Drosophila* contains a single gene that encodes a kinase that is highly homologous to PDK1 in its primary sequence and its domain structure (2). Initially, we identified two EP transposable elements in the 5′ region of the endogenous *Drosophila PDK1* gene *dPDK1* (Fig. 1*a*). These EP elements drive expression of dPDK1 under the control of the *Gal4* system (25, 34), allowing us to test whether dPDK1 and dAkt cooperate in promoting

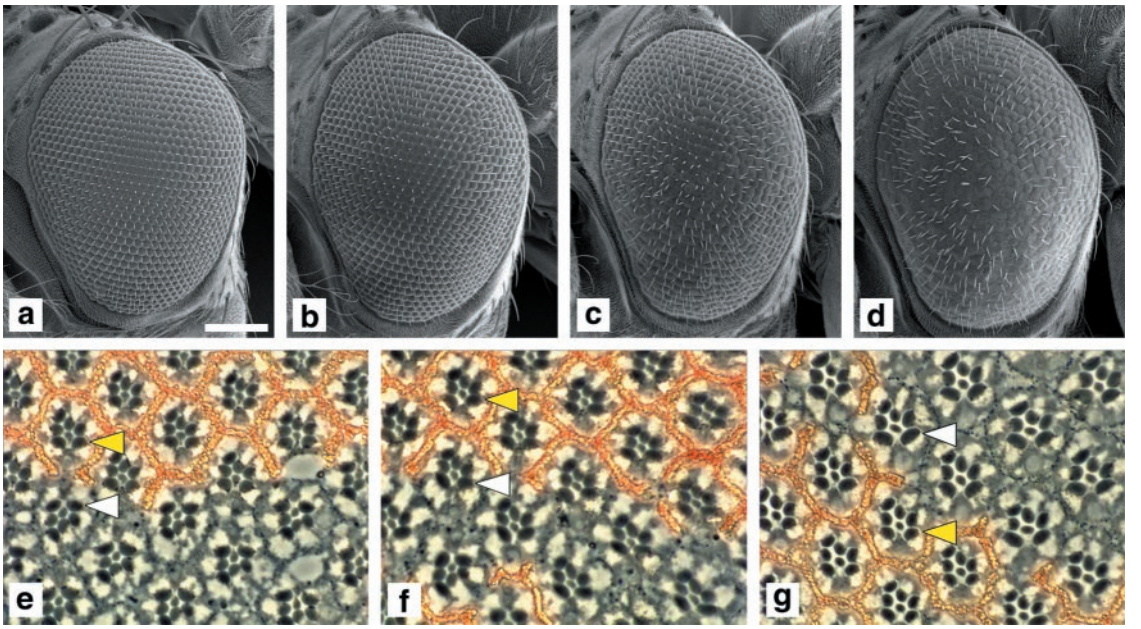

Fig. 2. Simultaneous overexpression of dPDK1 and dAkt with *GMR-Gal4* increases eye and cell size. (*a–d*) Simultaneous overexpression of dPDK1 and dAkt in the developing third instar eye imaginal disk results in the formation of larger eyes. Scanning electron micrographs of adult eyes of the following genotypes: (*a*) *OregonR*, wild type; (*b*) *y w*; *GMR-Gal4*/+; *EP(3)0837*/+; (*c*) *y w*; *GMR-Gal4 UAS-dAkt*/+; *TM2*/+; (*d*) *y w*; *GMR-Gal4 UAS-dAkt*/+; *TM2*/*EP(3)0837*. Although the overexpression of dPDK1 or dAkt alone results only in a slight, but in the case of dPDK1, significant increase in eye size (*b* and *c*), simultaneous expression of dPDK1 and dAkt causes a substantial increase in eye size (*d*). The area of at least 29 ommatidia in 3–6 eyes was measured for each genotype. Because the size of ommatidia of the genotypes *y w*; *GMR-Gal4 UAS-dAkt*/+; *TM2*/+ and *y w*; *GMR-Gal4 UAS-dAkt*/+; *TM2*/*EP(3)0837* is variable, only the values of the 30% largest ommatidia were included in the calculation. We used flies of the following genotype, *y w*; *GMR-Gal4*/*UAS-lacZ*, as a control. The means of these values are (normalized to a value of 100 ± SD): 100 ± 3 (control); 113 ± 3 (*b*); 108 ± 6 (*c*); 131 ± 10 (*d*). (*e–g*) dPDK1 and dAkt act synergistically to increase cell size in a cell-autonomous manner. Tangential sections through adult eyes containing clones in which dPDK1 and/or dAkt were overexpressed: (*e*) *y w hs-Flp*/*y w*; *GMR*>*FRT w*$^{+}$ STOP *FRT*>*Gal4*; *EP(3)0837*/+; (*f*) *y w hs-Flp*/*y w*; *GMR*>*FRT w*$^{+}$ STOP *FRT*>*Gal4*/*UAS-dAkt*; (*g*) *y w hs-Flp*/*y w*; *GMR*>*FRT w*$^{+}$ STOP *FRT*>*Gal4*/*UAS-dAkt*; *EP(3)0837*/+. Clones are marked by the lack of red pigment. No increase in cell size is observed by overexpressing dPDK1 or dAkt alone (*e* and *f*), but simultaneous overexpression of dPDK1 and dAkt slightly increases cell size (*g*). For quantification, the area of the R6 rhabdomere for 15 photoreceptors in clones overexpressing dPDK1 and/or dAkt (white arrowhead) were compared with the corresponding value for the sister control clone in the same section (yellow arrowhead): The values were normalized to 100 ± SD for the sister control clone and compared with the value in the overexpression clone: 100 ± 6 vs. 110 ± 7 (*e*); 100 ± 7 vs. 113 ± 7 (*f*); 100 ± 6 vs. 155 ± 10 (*g*). At the border of the clones, ommatidia composed of wild-type cells and cells overexpressing dPDK1 and/or dAkt are visible, indicating that the increase in cell size in *g* is cell-autonomous. (Bar = 100 μm.)

growth in *Drosophila*. Overexpression of either kinase in the eye imaginal disk during the last cell division cycle and subsequent differentiation showed little effect on the size or the structure of the eye (Fig. 2 *b* and *c*). Co-overexpression of dAkt and dPDK1, however, led to a significant increase in eye size (Fig. 2*d*). Furthermore, analysis of clones of cells in the eye overexpressing dPDK1 and/or dAkt revealed that the observed effect on cell size is strictly autonomous (Fig. 2 *e–g*). These results indicate that overexpression of dPDK1 does not interfere with the normal differentiation of eye disk cells and that it promotes local growth through dAkt activation.

To generate loss-of-function alleles of *dPDK1*, the dominant eye size phenotype caused by co-overexpression of dPDK1 and dAkt was reverted by using EMS mutagenesis, leading to three partial or complete loss-of-function mutations. *dPDK1*^{3} causes a G(352) to S substitution in the conserved DFG motif in the kinase subdomain VII (Fig. 1 *a* and *b*). The D residue in this motif is essential for kinase activity by orienting the ATP-Mg^{2+} complex for phosphotransfer (35–37). *dPDK1*^{4} causes a P(441) to L substitution in a conserved residue in kinase subdomain VIII. In the *dPDK1*^{5} allele, a Q codon at position 437 in kinase subdomain VIII is mutated to a STOP codon. Because this latter mutation results in the formation of a truncated dPDK1 protein lacking part of the kinase domain and the Pleckstrin-homology domain, *dPDK1*^{5} is likely to be a null mutation. A fourth allele *EP(3)3091* (*dPDK1*^{1}), from the Berkeley *Drosophila* Genome Project, has an EP element located in the third intron of *dPDK1* (Fig. 1*a*) and is homozygous lethal. It failed to complement *dPDK1*^{5} (data not shown), and the lethality was reversed by EP element excision.

Combinations of loss-of-function alleles provided mutants of varying strengths. Larvae homozygous for the *dPDK1*^{5} null allele or larvae of the *dPDK1*$^{1/5}$ heteroallelic combination die during the second instar stage. A less severe reduction in dPDK1 function (*dPDK1*$^{4/5}$) permits development of viable *dPDK1* mutant flies that are delayed 1 day in development and smaller than their heterozygous siblings, having an 18% reduction in body weight (Fig. 3 *a* and *d*). By measuring the cell density in the wing, the reduction in size and weight apparently is primarily caused by a decrease in cell size, because cell number is only slightly affected (Fig. 3*d*). The lethality associated with the *dPDK1* null allele and the size defect of *dPDK1* hypomorphs was rescued by ubiquitous expression of a wild-type *dPDK1* transgene with *armadillo (arm)-Gal4* as a driver. *dPDK1*$^{4/5}$ male flies are almost completely sterile, although they show no obvious defect in sperm morphology and motility and in mating behavior (data not shown). That loss of zygotic dPDK1 function results in larval lethality is in contrast to a recent analysis of two *dPDK1* mutations caused by the EP insertion *EP(3)3091* (*dPDK1*^{1}) or a 10-kb deletion (*dPDK1*^{2}), which were homozygous embryonic lethal (19). It is possible that the embryonic lethality observed by Cho *et al.* (19) is not caused by loss of dPDK1 function but by a linked lethal mutation on the same chromosome, because no rescue was attempted, and the phenotype was only analyzed in

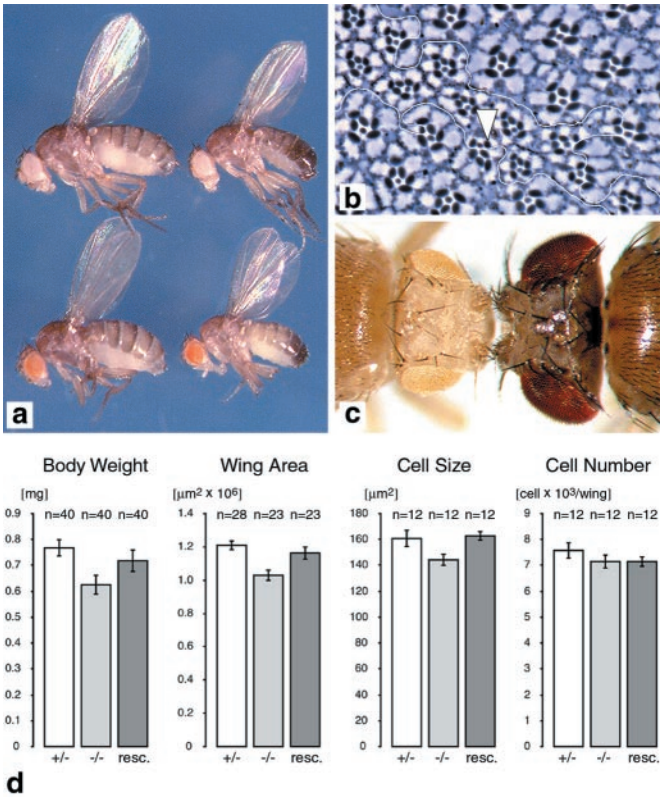

Fig. 3. dPDK1 loss-of-function phenotypes. (*a*) Body size reduction of heteroallelic mutant flies. Males (*Right*) and females (*Left*) of the following genotypes are shown: *y w*; *dPDK1*^{5}/+ (*Top*); *y w*; *dPDK1*^{4}/*dPDK1*^{5} (*Bottom*). (*b*) Tangential section through an eye containing a *dPDK1*$^{5/5}$ clone. Within the clone, all photoreceptor cells are reduced in size compared with wild-type photoreceptor cells. At the border of the clone, ommatidia composed of phenotypically wild-type and mutant cells (arrowhead) are visible, indicating that dPDK1 controls cell size autonomously. The genotype is as follows: *y w ey-Flp*/*y w*; *dPDK1*^{5} *FRT80B*/*FRT80B*. (*c*) Selective removal of dPDK1 function from the eye imaginal disk results in a reduction of head and eye size. *y w ey-Flp*/*y w*; *dPDK1*^{5} *FRT80B*/*M(3)67c*^{4} *FRT80B* (*Left*); *OregonR*, wild type (*Right*). (*d*) Quantification of body and organ size in *dPDK1* heteroallelic mutant male flies compared with heterozygous and rescued flies, which overexpress a wild-type *dPDK1* cDNA under the control of the ubiquitous *arm-Gal4* driver. Values of *y w*; *dPDK1*^{5}/+ (+/−), *y w*; *dPDK1*^{4}/*dPDK1*^{5} (−/−), and *y w*; *arm-Gal4*/*UAS-dPDK1*; *dPDK1*^{4}/*dPDK1*^{5} flies (resc.) are shown. Values are the mean ± SD.

homozygotes. Consistent with this observation, larvae homozygous for a *dPDK1*^{1} mutant chromosome, which has been cleaned from second hits by recombination, die during the second instar stage. Although it is very likely that dPDK1 functions during embryogenesis, like dAkt (33), maternal transcripts may be sufficient to support embryonic development.

To determine whether the effects of loss of dPDK1 function on cell growth and organ development are autonomous events, we analyzed loss of dPDK1 in clones of cells by using the *FRT* mitotic recombination system (22). In contrast to organism lethality, clones of cells homozygous for the *dPDK1* null allele *dPDK1*^{5} survive to adulthood. These cells show no defect in their ability to differentiate into photoreceptor cells or accessory cells, but mutant photoreceptor cells are ≈30% smaller than the heterozygous cells outside the clone (Fig. 3*b*), a strictly cell autonomous effect. To test whether an entire body part could develop in the absence of dPDK1 function, dPDK1 was selectively removed in much of the head primordium by using the *ey-Flp* system (23). Heads homozygous mutant for anyone of the three alleles, *dPDK1*^{3}, *dPDK1*^{4}, and *dPDK1*^{5}, are reduced in size (Fig. 3*c*; data not shown), which indicates that entire organs differentiate and develop in the absence of dPDK1 function, but that the final size of these organs autonomously depends on the amount of dPDK1 activity. The reduction in head size was most severe with *dPDK1*^{5} followed by *dPDK1*^{4} and *dPDK1*^{3}, with the complete removal of dPDK1 function similar to that observed for loss-of-function mutations in the *Drosophila* insulin receptor (*dInr*), *Dp110*/*PI(3)K,* and *dAkt* (ref. 27; H.S. and E.H., unpublished work).

The pronounced effect of loss of dPDK1 function on head size suggested that it is a dominant constituent in the dInr pathway. To test this possibility, we examined the ability of complete and partial loss-of-function alleles of *dPDK1* to reverse phenotypes caused by either overexpression of dInr or by mutations in *dPTEN*, the 3-phosphatidylinositide phosphatase. Overexpression of a wild-type *dInr* cDNA under the control of *GMR-Gal4* led to a marked increase in eye size and a slightly rough eye surface (27), an effect dominantly suppressed by removing one copy of *dPDK1* (Fig. 4*a*). Further reduction of dPDK1 function by the *dPDK1*$^{1/4}$ heteroallelic combination reduced the eye to almost wild-type size (Fig. 4*b*), suggesting that the amount of dPDK1 protein is rate-limiting for the dInr overgrowth phenotype. Null mutations in *dPTEN* cause lethality, and removal of dPTEN function in clones stimulates cell autonomous growth (30, 31, 38), suggesting that increased levels of PIP_3 promote growth and are the likely cause of lethality. Thus, if dPDK1 is an essential target of PIP_3, mutations in *dPDK1* may suppress the *dPTEN* phenotype. Surprisingly, some *dPTEN*/*dPDK1* double mutant flies survive to adulthood (Fig. 4*c*), indicating that the presumed PIP_3-induced lethality is primarily caused by the hyperactivation of dPDK1 or of one of its targets.

The fact that the growth phenotypes of *dPDK1* mutations are similar to those caused by mutations in genes coding for dS6K (32), and dAkt (refs. 39 and 40; H.S. and E.H., unpublished work), and that S6K1 is a mammalian PDK1 substrate, raised the possibility that dPDK1 may independently control growth through dS6K. This possibility was tested in the wing, which is composed of a dorsal and a ventral epithelial sheet that are tightly attached to each other through extracellular matrix. We have shown that selective overexpression of a wild-type *dS6K* cDNA in the dorsal wing epithelium with the *apterous* (*ap*)-*Gal4* driver leads to a bending down of the wing blade, probably because of a cell-size increase in the dorsal surface (32). This phenotype was suppressed by a reduction of dPDK1 function (Fig. 5 *a–c*). Although *ap-Gal4* induced overexpression of wild-type dPDK1 alone had little effect on wing morphology (data not shown), overexpression of a dPDK1^{A467V} variant was sufficient to cause a bent-wing phenotype (Fig. 5*d*). The corresponding amino acid substitution in the *C. elegans* PDK1 is thought to cause a hyperactivation of the kinase (18). The dPDK1^{A467V}-induced bent wing phenotype depends on normal levels of dS6K and dAkt, because null mutations in either of the corresponding genes dominantly suppress the phenotype (Fig. 5 *e* and *f*). Together with the biochemical evidence in cultured cells and *in vivo* that dPDK1 controls the activity of dAkt and dS6K (ref. 19; T. Radimerski, J. Montagne, F. R. J. van der Kaay, C. P. Downes, E.H., and G.T., unpublished work) these results provide functional evidence that dPDK1 is a key regulator in the control of growth and cell size by regulating the activity of two AGC kinases, dAkt and dS6K.

The effects of dPDK1 on dS6K raised the possibility that dPDK1 controls the activity of other AGC kinases *in vivo*, such as dRSK and dPKN, which have been implicated as mammalian PDK1 substrates. Because the developing eye depends on endogenous levels of dPDK1, we examined whether lowering the dose of dPDK1 was sufficient to suppress dominantly the rough eye phenotype caused by overexpression of dRSK and dPKN under *GMR-Gal4* control (Fig. 5*g*; data not shown). Reduction of dPDK1 activity in a viable *dPDK1* mutant combination was sufficient to suppress the rough eye phenotype of dRSK but not

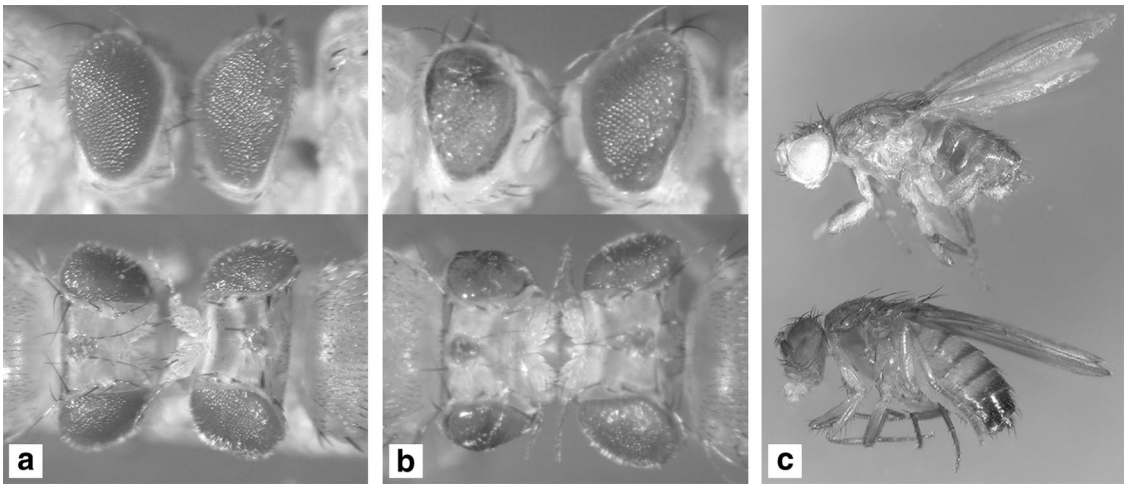

Fig. 4. dPDK1 loss-of-function mutations suppress *dInr* and *dPTEN* mutant phenotypes. (*a* and *b*) The eye phenotype caused by overexpression of *UAS-dInr* with the *GMR-Gal4* driver is dominantly suppressed by removing one copy of *dPDK1*, and the eye size is almost completely restored to wild-type size in a $dPDK1^{1/4}$ heteroallelic mutant background, although eye roughness is increased. The reason for this latter observation is unclear. (*a*) *y w*; *GMR-Gal4 UAS-dInr*/+; $dPDK1^{5}$/+ (*Left*); *y w*; *GMR-Gal4 UAS-dInr*/+; *MKRS*/+ (*Right*); (*b*) *y w*; *GMR-Gal4 UAS-dInr*/+; $dPDK1^{1}/dPDK1^{4}$ (*Left*); *y w*; *GMR-Gal4 UAS-dInr*/+; $dPDK1^{4}$/+ (*Right*). (*c*) The lethality caused by mutations in *dPTEN* is rescued in a *dPDK1* heteroallelic mutant background: Some *dPTEN*, *dPDK1* double-mutant flies survive to adulthood, although they display mutant phenotypes like an unproportionally reduced size of the abdomen and deformed leg structures. Similar phenotypes have been observed in partial loss-of-function mutations for *dTOR* (S. Oldham and E.H., unpublished work). Flies of the following genotypes are shown: *y w* $dPTEN^{dj189}/dPTEN^{494}$; $dPDK1^{4}/dPDK1^{5}$ (*Upper*), *OregonR,* wild-type (*Lower*).

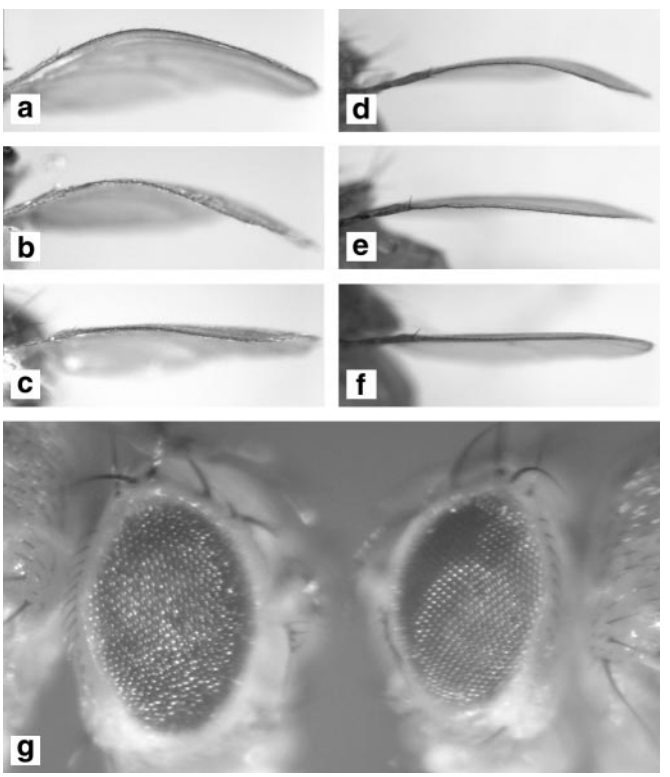

Fig. 5. Genetic interaction of dPDK1 with the AGC kinases dAkt, dS6K, and dRSK. (*a–c*) Mutations in *dPDK1* suppress the *ap-Gal4 UAS-dS6K* bent-wing phenotype. (*a*) *y w*; *ap-Gal4 UAS-dS6K*/+; *MKRS*/+; (*b*) *y w*; *ap-Gal4 UAS-dS6K*/+; $dPDK1^{5}$/+; (*c*) *y w*; *ap-Gal4 UAS-dS6K*/+; $dPDK1^{4}/dPDK1^{5}$. (*d–f*) Null mutations in *dS6K* and *dAkt* dominantly suppress the *ap-Gal4 UAS-*$dPDK1^{A467V}$ bent-wing phenotype. (*d*) *y w*; *ap-Gal4 UAS-*$dPDK1^{A467V}$/+; (*e*) *y w*; *ap-Gal4 UAS-*$dPDK1^{A467V}$/+; $dS6K^{l-1}$/+; (*f*) *y w*; *ap-Gal4 UAS-*$dPDK1^{A467V}$/+; $dAkt^{1}$/+. (*g*) Mutations in *dPDK1* suppress the rough eye phenotype caused by overexpression of *UAS-dRSK* under *GMR-Gal4* control. *y w*; *GMR-Gal4 UAS-dRSK*/+; $dPDK1^{5}$/+ (*Left*); *y w*; *GMR-Gal4 UAS-dRSK*/+; $dPDK1^{4}/dPDK1^{5}$ (*Right*).

of dPKN overexpression (Fig. 5*g*; data not shown). These results suggest that at least in this *in vivo* assay, dRSK activity critically depends on dPDK1 function, whereas dPKN activity is not changed by a reduction in dPDK1 levels. This idea is in line with the recent finding that in $PDK1^{-/-}$ embryonic stem cells the protein kinase C-related protein kinase PRK2, which shares extensive homology with PKN, is still partially phosphorylated at its T loop residue (41), indicating that PDK1-independent mechanisms for the phosphorylation of the T loop of certain AGC kinases including dPKN may exist.

Our results show that dPDK1 is an essential component in the insulin signaling pathway in the control of cell growth and body size through its two substrates, dAkt and dS6K. These results are distinct from the genetic evidence in *C. elegans* where Akt is the primary target of PDK1 in dauer formation. Because mutations in the insulin signaling pathway do not show an autonomous alteration of cell size in *C. elegans*, the regulation of the rate of protein synthesis through S6K does not seem to be a primary target of this pathway. However, that dPDK1 may yet have additional substrates is suggested by the genetic interaction with *dRSK* gain-of-function mutations and because viable *dPDK1* males are almost completely sterile. Although mutations in components of the insulin signaling pathway such as *dInr*, *chico*, *Dp110/PI(3)K*, and *dAkt* cause female sterility, male sterility is not observed. Further genetic dissection of dPDK1 function is required to determine the role of dPDK1 in male fertility. Our findings in *Drosophila* are consistent with the absence of insulin growth factor-1-induced activation of S6K, Akt, and RSK in mammalian $PDK1^{-/-}$ embryonic stem cells (17), and therefore provide evidence for the functional conservation of branch points in kinase networks during evolution.

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1. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. & Cohen, P. (1997) *Curr. Biol.* **7,** 261–269.
2. Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbison, D., *et al*. (1997) *Curr. Biol.* **7,** 776–789.
3. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F. & Hawkins, P. T. (1997) *Science* **277,** 567–570.
4. Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G. F., Holmes, A. B., Gaffney, P. R., Reese, C. B., McCormick, F., Tempst, P., *et al*. (1998) *Science* **279,** 710–714.
5. Belham, C., Wu, S. & Avruch, J. (1999) *Curr. Biol.* **9,** R93–R96.
6. Peterson, R. T. & Schreiber, S. L. (1999) *Curr. Biol.* **9,** R521–R524.
7. Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N. & Avruch, J. (1998) *Curr. Biol.* **8,** 69–81.
8. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A. & Thomas, G. (1998) *Science* **279,** 707–710.
9. Jensen, C. J., Buch, M. B., Krag, T. O., Hemmings, B. A., Gammeltoft, S. & Frodin, M. (1999) *J. Biol. Chem.* **274,** 27168–27176.
10. Frodin, M., Jensen, C. J., Merienne, K. & Gammeltoft, S. (2000) *EMBO J.* **19,** 2924–2934.
11. Dong, L. Q., Landa, L. R., Wick, M. J., Zhu, L., Mukai, H., Ono, Y. & Liu, F. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 5089–5094. (First Published May 2, 2000; 10.1073/pnas.090491897)
12. Dutil, E. M., Toker, A. & Newton, A. C. (1998) *Curr. Biol.* **8,** 1366–1375.
13. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P. & Parker, P. J. (1998) *Science* **281,** 2042–2045.
14. Chou, M. M., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. S. & Toker, A. (1998) *Curr. Biol.* **8,** 1069–1077.
15. Dong, L. Q., Zhang, R. B., Langlais, P., He, H., Clark, M., Zhu, L. & Liu, F. (1999) *J. Biol. Chem.* **274,** 8117–8122.
16. Anderson, K. E., Coadwell, J., Stephens, L. R. & Hawkins, P. T. (1998) *Curr. Biol.* **8,** 684–691.
17. Williams, M. R., Arthur, J. S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P. & Alessi, D. R. (2000) *Curr. Biol.* **10,** 439–448.
18. Paradis, S., Ailion, M., Toker, A., Thomas, J. H. & Ruvkun, G. (1999) *Genes Dev.* **13,** 1438–1452.
19. Cho, K. S., Lee, J. H., Kim, S., Kim, D., Koh, H., Lee, J., Kim, C., Kim, J. & Chung, J. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 6144–6149. (First Published May 8, 2001; 10.1073/pnas.101596998)
20. Lewis, E. B. & Bacher, F. (1968) *Drosophila Inf. Serv.* **43,** 193–194.
21. MacDougall, C. N., Clyde, D., Wood, T., Todman, M., Harbison, D. & Bownes, M. (1999) *Eur. J. Biochem.* **262,** 456–466.
22. Xu, T. & Rubin, G. M. (1993) *Development (Cambridge, U.K.)* **117,** 1223–1237.
23. Newsome, T. P., Asling, B. & Dickson, B. J. (2000) *Development (Cambridge, U.K.)* **127,** 851–860.
24. Basler, K. & Hafen, E. (1988) *Cell* **54,** 299–311.
25. Brand, A. H. & Perrimon, N. (1993) *Development (Cambridge, U.K.)* **118,** 401–415.
26. Basler, K., Christen, B. & Hafen, E. (1991) *Cell* **64,** 1069–1081.
27. Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R. & Hafen, E. (2001) *Curr. Biol.* **11,** 213–221.
28. Hamilton, B. A., Palazzolo, M. J., Chang, J. H., VijayRaghavan, K., Mayeda, C. A., Whitney, M. A. & Meyerowitz, E. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2731–2735.
29. Calleja, M., Moreno, E., Pelaz, S. & Morata, G. (1996) *Science* **274,** 252–255.
30. Gao, X., Neufeld, T. P. & Pan, D. (2000) *Dev. Biol.* **221,** 404–418.
31. Huang, H., Potter, C. J., Tao, W., Li, D. M., Brogiolo, W., Hafen, E., Sun, H. & Xu, T. (1999) *Development (Cambridge, U.K.)* **126,** 5365–5372.
32. Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C. & Thomas, G. (1999) *Science* **285,** 2126–2129.
33. Staveley, B. E., Ruel, L., Jin, J., Stambolic, V., Mastronardi, F. G., Heitzler, P., Woodgett, J. R. & Manoukian, A. S. (1998) *Curr. Biol.* **8,** 599–602.
34. Rorth, P. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 12418–12422.
35. Moran, M. F., Koch, C. A., Sadowski, I. & Pawson, T. (1988) *Oncogene* **3,** 665–672.
36. Taylor, S. S., Knighton, D. R., Zheng, J., Sowadski, J. M., Gibbs, C. S. & Zoller, M. J. (1993) *Trends Biochem. Sci.* **18,** 84–89.
37. van der Geer, P., Hunter, T. & Lindberg, R. A. (1994) *Annu. Rev. Cell Biol.* **10,** 251–337.
38. Goberdhan, D. C., Paricio, N., Goodman, E. C., Mlodzik, M. & Wilson, C. (1999) *Genes Dev.* **13,** 3244–3258.
39. Verdu, J., Buratovich, M. A., Wilder, E. L. & Birnbaum, M. J. (1999) *Nat. Cell Biol.* **1,** 500–506.
40. Scanga, S. E., Ruel, L., Binari, R. C., Snow, B., Stambolic, V., Bouchard, D., Peters, M., Calvieri, B., Mak, T. W., Woodgett, J. R., *et al*. (2000) *Oncogene* **19,** 3971–3977.
41. Balendran, A., Hare, G. R., Kieloch, A., Williams, M. R. & Alessi, D. R. (2000) *FEBS Lett.* **484,** 217–223.
42. Clyde, D. & Bownes, M. (2000) *J. Endocrinol.* **167,** 391–401.

The *Drosophila* Forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling

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The *Drosophila* Forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling

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Abstract

Background: Forkhead transcription factors belonging to the FOXO subfamily are negatively regulated by protein kinase B (PKB) in response to signaling by insulin and insulin-like growth factor in *Caenorhabditis elegans* and mammals. In *Drosophila*, the insulin-signaling pathway regulates the size of cells, organs, and the entire body in response to nutrient availability, by controlling both cell size and cell number. In this study, we present a genetic characterization of *dFOXO*, the only *Drosophila FOXO* ortholog.

Results: Ectopic expression of *dFOXO* and human *FOXO3a* induced organ-size reduction and cell death in a manner dependent on phosphoinositide (PI) 3-kinase and nutrient levels. Surprisingly, flies homozygous for *dFOXO* null alleles are viable and of normal size. They are, however, more sensitive to oxidative stress. Furthermore, dFOXO function is required for growth inhibition associated with reduced insulin signaling. Loss of dFOXO suppresses the reduction in cell number but not the cell-size reduction elicited by mutations in the insulin-signaling pathway. By microarray analysis and subsequent genetic validation, we have identified *d4E-BP*, which encodes a translation inhibitor, as a relevant dFOXO target gene.

Conclusion: Our results show that dFOXO is a crucial mediator of insulin signaling in *Drosophila*, mediating the reduction in cell number in insulin-signaling mutants. We propose that in response to cellular stresses, such as nutrient deprivation or increased levels of reactive oxygen species, dFOXO is activated and inhibits growth through the action of target genes such as *d4E-BP*.

Background

Receptors for insulin and insulin-like growth factors (IGFs) are central regulators of energy metabolism and organismal growth in vertebrates and invertebrates. In mammals, the insulin receptor regulates glucose homeostasis and embryonic growth [1], whereas the insulin-like growth factor 1 receptor (IGF1-R) regulates embryonic and postembryonic growth [2] and longevity [3]. In *Caenorhabditis elegans*, DAF-2 - the homolog of the mammalian insulin/IGF receptor - controls organismal growth in response to poor nutrient conditions indirectly by controlling formation of the long-lived, stress-resistant dauer stage during larval development, and lifespan in the adult [4]. In *Drosophila*, the insulin/IGF receptor homolog DInr controls organismal growth directly by regulating cell size and cell number [5]. Furthermore, reduced insulin signaling causes female sterility and independently increases lifespan [6,7]. The striking conservation of insulin receptor function is also reflected in the conservation of the intracellular signaling cascade. Binding of insulin-like peptides to their receptor tyrosine kinases leads to the activation of class I_A phosphatidylinositol (PI) 3-kinases and increased intracellular concentrations of the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). This results in recruitment to the membrane, and activation, of the protein kinases phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (PKB/AKT), both of which contain pleckstrin homology (PH) domains and which in turn modulate the activity of downstream effector proteins [8]. The lipid phosphatase PTEN (phosphatase and tensin homolog on chromosome 10) catalyzes the 3-dephosphorylation of PIP_3, thereby acting as a negative regulator of insulin signaling [9]. The demonstration that the lethality associated with loss of dPTEN in *Drosophila* is rescued by a mutant form of dPKB with impaired affinity for PIP_3 indicates that PKB is a key effector of this pathway [10]. Genetic and biochemical studies have identified two critical targets of PKB, namely forkhead transcription factors of the FOXO subfamily and the Tuberous Sclerosis Complex 2 (TSC2) tumor suppressor protein.

In *C. elegans*, the only FOXO transcription factor is encoded by *daf-16*. Loss-of-function mutations in *daf-16* completely suppress the dauer-constitutive and longevity phenotypes associated with reduced function of insulin-signaling components. On the basis of knowledge about DAF signaling in *C. elegans*, forkhead transcription factors belonging to the FOXO subfamily have been identified as direct targets of insulin/IGF signaling in mammals [11-13]. The mammalian DAF-16 homologs comprise the proteins FOXO1 (FKHR), FOXO3a (FKHRL1) and FOXO4 (AFX). Their phosphorylation by the insulin-activated kinases PKB and serum- and glucocorticoid-regulated protein kinase (SGK) creates binding sites for 14-3-3 proteins, and this leads to inactivation of FOXO proteins via cytoplasmic sequestration [12,14]. The result of this process is an insulin-induced transcriptional repression of FOXO target genes, which are involved in the response to DNA damage [15] and oxidative stress [16,17], apoptosis [12,18], cell-cycle control [19-21] and metabolism [22]. In addition to their transcriptional activation capabilities, FOXO proteins have recently been shown to induce cell-cycle arrest by repressing transcription of genes encoding D-type cyclins [23,24]. FOXO transcription factors mediate insulin resistance in diabetic mice [25], and have been proposed to be tumor suppressors, as several chromosomal translocations disrupting FOXO genes are found in cancers [26,27], and overexpressed FOXO proteins can inhibit tumor growth [23].

TSC2, the second target of PKB, forms a complex with TSC1 and acts as a negative regulator of growth in *Drosophila*, and as a tumor suppressor in mammals. Overexpressed activated PKB phosphorylates TSC2 and thereby disrupts the TSC1/2 complex in *Drosophila* and in mammalian cells [28,29]. In *Drosophila*, the TSC1/2 complex functions by negatively regulating two kinases, dTOR (homolog of the mammalian target of rapamycin) [30] and dS6K (homolog of the mammalian ribosomal protein S6 kinase) [31]. Recent genetic and biochemical evidence indicates that TSC1/2 regulates S6K activity by acting as a GTPase-activating protein (GAP) for the small GTPase Rheb [32-35]. Interestingly, flies lacking dS6K function are reduced in size because of a reduction in cell size but not in cell number [36]. The growth control pathways regulating cell size and cell number therefore bifurcate either at dPKB or between dPKB and dS6K.

In this study, we describe the identification of dFOXO, the single FOXO ortholog in *Drosophila*. Although dFOXO function is not essential for development and organismal growth control under normal culture conditions, it mediates the reduction in cell number associated with reduced insulin signaling. Our results show that dFOXO regulates expression of *d4E-BP*, which mediates part of the cell-number reduction in *dPKB* mutants. We propose that dFOXO upregulates *d4E-BP* transcription under conditions of low insulin signaling. Furthermore, our observations suggest that dFOXO is required for resistance against oxidative stress in adult flies.

Results

dFOXO is the only *Drosophila* homolog of FOXO and DAF-16

The *Drosophila* genome contains a single homolog of the DAF-16/FOXO family of transcription factors. This notion is

supported by the phylogenetic tree diagram calculated from the multiple sequence alignment (Figure 1a). The *dFOXO* gene is more closely related to the mammalian *FOXO* subfamily and *daf-16* than any other *Drosophila* forkhead gene. The amino-acid sequences of the predicted 613 amino-acid dFOXO protein and hFOXO3a are 27% identical over the full protein length, and 82% identical within the forkhead DNA-binding domain. Furthermore, *dFOXO* is the only *Drosophila* forkhead gene encoding a putative protein containing conserved PKB phosphorylation sites [37]. The orientation of the three PKB consensus sites relative to the forkhead domain (Figure 1b) is conserved among the mammalian FOXO

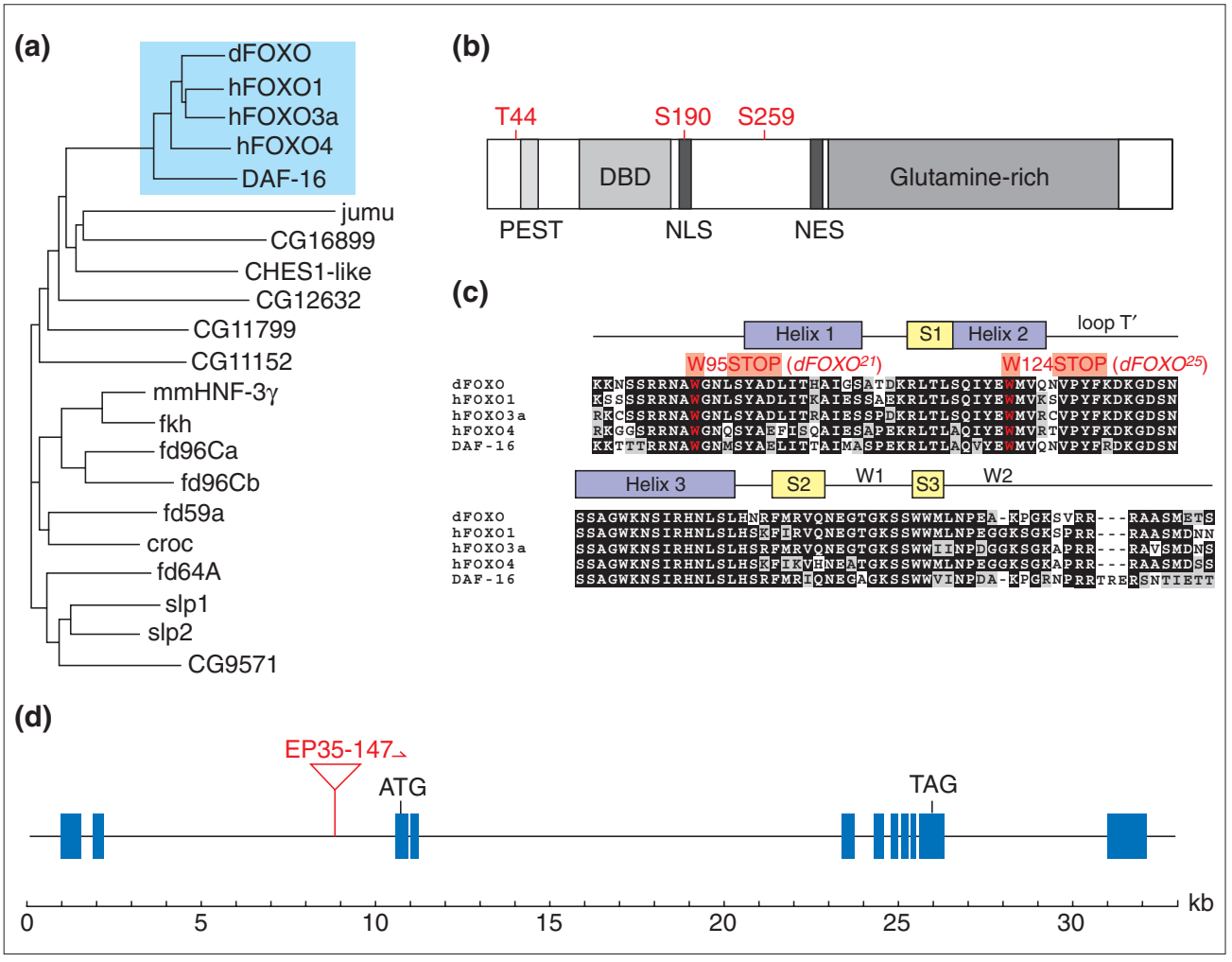


Figure 1
dFOXO is the only *Drosophila* FOXO/DAF-16 homolog. A TBLASTN search of the *Drosophila* genome for known and predicted genes encoding forkhead transcription factors retrieved 16 genes. **(a)** A phylogenetic tree calculated from a multiple sequence alignment of the forkhead domains of these 16 proteins and of the human FOXO proteins FOXO1 (FKHR), FOXO3a (FKHRL1) and FOXO4 (AFX), the *C. elegans* DAF-16 and mouse Foxa3 (HNF-3γ; protein names on the figure are from GenBank). The similarity of dFOXO to FOXO proteins is highlighted in blue. **(b)** dFOXO has three PKB phosphorylation sites in the same orientation as those of mammalian FOXO proteins. The sites are indicated above the protein; PEST (destruction), nuclear localization (NLS), nuclear export (NES) and DNA-binding sequences are also shown. **(c)** A multiple amino-acid sequence alignment of the dFOXO, human FOXO and DAF-16 forkhead domains illustrates the high degree of sequence conservation especially within the DNA-binding domain. The secondary structure is indicated above the alignment. Similar and identical amino-acid residues are shaded in gray and black, respectively. The region encoding helix 3 of the forkhead domain, which is the DNA-recognition helix contacting the major groove of the DNA double helix, is identical in the five proteins. Given the high structural similarity between the DNA-binding domains of FOXO4 (AFX) and HNF-3γ [86], it is likely that FOXO proteins contact insulin response elements through helix 3. Two EMS-induced point mutations described in this study are shown in red. **(d)** The *dFOXO* gene spans a genomic region of 31 kilobases (kb) and contains 11 exons (blue bars). The *EP35-147* transposable element is inserted in the second intron upstream of the open reading frame, allowing GAL4-induced expression of endogenous *dFOXO*.

proteins, DAF-l6 and dFOXO. Figure 1c shows the high degree of sequence conservation between dFOXO and FOXO/DAF-16 proteins within the DNA-binding domain. Taken together, these observations strongly suggest that dFOXO is the only *Drosophila* homolog of the mammalian FOXO transcription factors and *C. elegans* DAF-l6.

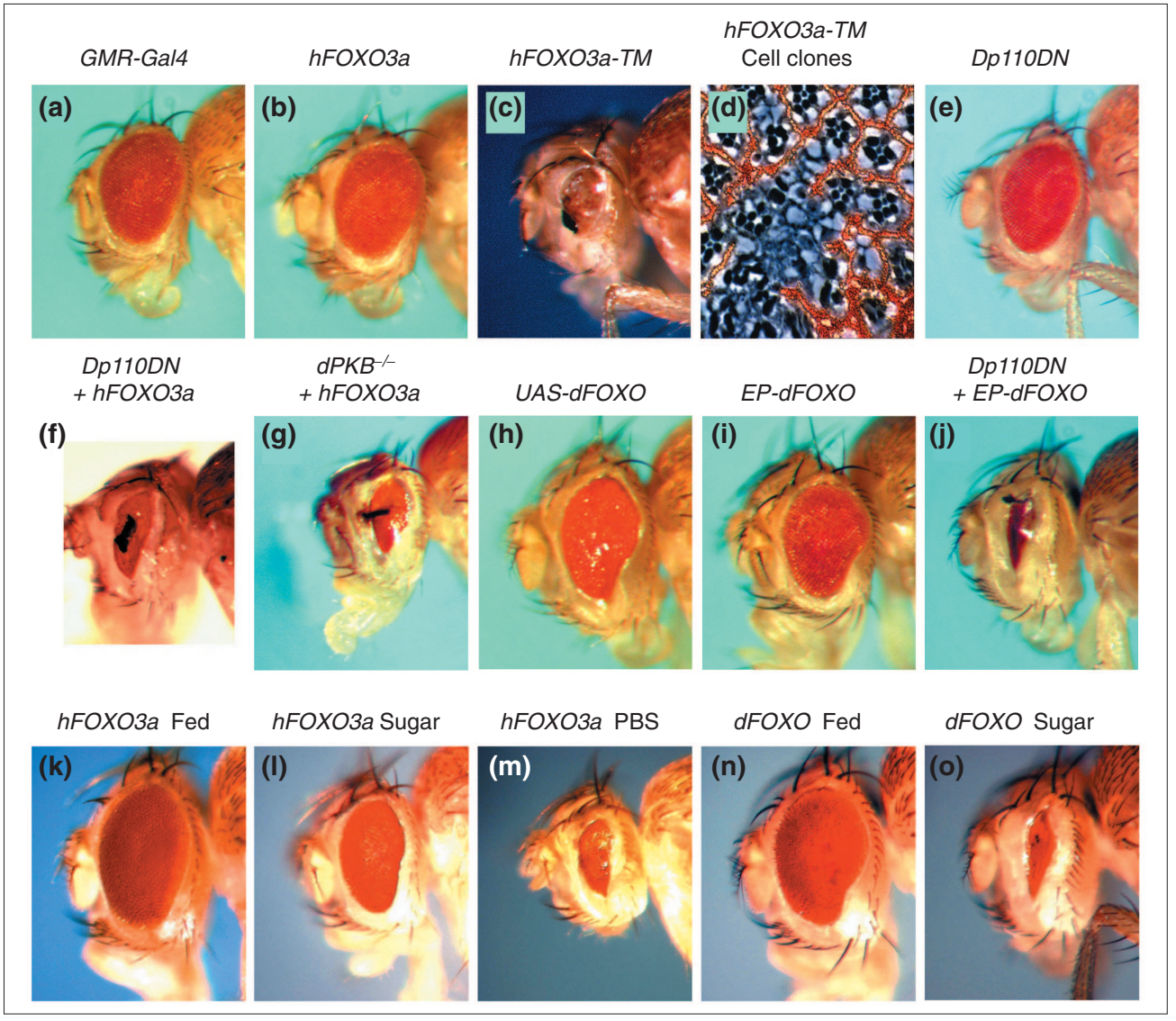


Figure 2
Targeted *hFOXO3a* and *dFOXO* expression in the developing *Drosophila* eye induces organ-size reduction and cell death, and the phenotypes are sensitive to insulin signaling and nutrient levels. **(a)** *GMR-Gal4*-expressing control fly. **(b)** No discernible phenotype results from *hFOXO3a* expression. **(c)** Expression of *hFOXO3a-TM* in the eye disc leads to pupal lethality; escapers at 18°C show a necrotic phenotype and severely disrupted cell specification. **(d)** Expression in *w*$^{-}$-marked clones of cells induces a similar phenotype at 25°C. **(e)** *Dp110DN* expression slightly reduces eye size, and **(f)** co-expression of wild-type *hFOXO3a* partially mimicks the *hFOXO3a-TM* escaper phenotype. **(g)** The same enhancement of hFOXO3a activity was observed in a *dPKB*$^{-/-}$ background. **(h,i)** Expression of transgenic or endogenous *dFOXO* results in a small-eye phenotype, which is also dramatically enhanced by **(j)** *Dp110DN*. **(k-o)** *hFOXO3a* and *dFOXO* phenotypes are progressively exacerbated by protein deprivation ('sugar') and complete starvation ('PBS'). Flies like the one shown in (m) die within one day, and complete starvation of *dFOXO*-expressing flies resulted in pupal lethality (not shown). Genotypes are: (a) *y w; GMR-Gal4/+*; (b) *y w; GMR-Gal4/+; UAS-hFOXO3a/+*; (c) *y w; GMR-Gal4/+; UAS-hFOXO3a-TM/+*; (d) *y w hs-flp/y w; GMR > FRT- w*$^{+}$ *STOP - FRT > Gal-4/+; UAS-hFOXO3a-TM/+*; (e) *y w; GMR-Gal4 UAS-Dp110DN/+*; (f) *y w; GMR-Gal4 UAS-Dp110DN/+; UAS-hFOXO3a/+*; (g) *y w; UAS-hFOXO3a/GMR-Gal4; dPKB*^{3}*/dPKB*^{1}; (h) *y w; UAS-dFOXO/GMR-Gal4*; (i) *y w; GMR-Gal4/+; EP-dFOXO/+*; (j) *y w; GMR-Gal4 UAS-Dp110DN/+; EP-dFOXO/+*; (k-m) *y w; GMR-Gal4/+; UAS-hFOXO3a/+*; (n,o) *y w; GMR-Gal4/+; EP-dFOXO/+*.

Overexpressed dFOXO is responsive to insulin signaling and nutrient levels, inducing organ-size reduction and cell death

To assess whether dFOXO has a key function in insulin signaling like that of DAF-16 in *C. elegans*, we tested whether overexpression of wild-type or mutant forms of *hFOXO3a* and *dFOXO* could antagonize insulin signaling. Elimination of the three PKB consensus phosphorylation sites in mammalian FOXO3a prevents its phosphorylation, subsequent binding to 14-3-3 proteins, and sequestration in the cytoplasm [12]. This leads to constitutive nuclear localization of the mutant FOXO3a and transcriptional activation of its target genes. Assuming that blocking the PKB signal would have the same activating effect on dFOXO, we overexpressed wild-type and triple PKB-phosphorylation-mutant variants of both *dFOXO* and human *FOXO3a*. Furthermore, we identified an *EP* transposable element insertion in the second *dFOXO* intron, which permits the GAL4-induced overexpression of endogenous *dFOXO* (Figure 1d). We used the *GMR-Gal4* construct to drive UAS-dependent expression in postmitotic cells in the eye imaginal disc [38]. While expression of wild-type *hF0X03a* in the developing eye did not result in a visible phenotype (Figure 2b), *hFOXO3a-TM* expression caused pupal lethality. Few escaper flies eclosed and displayed a strong necrotic eye phenotype (Figure 2c). A block of cell differentiation and necrosis was also observed when *hFOXO3a-TM* was expressed in cell clones in the developing eye (Figure 2d).

Assuming that the lack of a phenotype observed upon *UAS-hFOXO3a* expression is due to hFOXO3a inactivation by endogenous DInr signaling in the eye disc, we performed the same experiment in a background of reduced insulin signaling. Indeed, in the presence of a dominant-negative (DN) form of Dp110 (encoding the PI 3-kinase catalytic subunit) [39], *hFOXO3a* expression induced a necrotic phenotype similar to the one observed with the hyperactive phosphorylation mutant (Figure 2f). To confirm that hFOXO3a is responsive to *Drosophila* insulin signaling and rule out artificial coexpression effects, we expressed *hFOXO3a* in flies mutant for either *dPKB* (Figure 2g) or *Dp110* (not shown), and observed similar phenotypes to those seen upon coexpression of *Dp110DN*. *Drosophila* FOXO has qualitatively similar, but stronger effects. Expressing the wild-type form of *dFOXO* causes a weak eye-size reduction and disruption of the ommatidial pattern even in a wild-type background (Figure 2h,i), and the phenotype is strongly affected by Dp110DN as well (Figure 2j). The *UAS-dFOXO-TM* transgene appears to cause lethality even in the absence of a *Gal4* driver, as we did not obtain viable transgenic lines with this construct. Furthermore, we examined the effects of nutrient deprivation on FOXO-expressing tissues. If nutrient availability is limited, FOXO should be more active in response to lowered insulin signaling. Indeed, we observed that the overexpression phenotypes of both *hFOXO3a* and *dFOXO* are enhanced under conditions of starvation. *Drosophila* larvae that are starved until 70 h after egg laying (AEL) die within a few days. But if the onset of nutrient deprivation occurs after they have surpassed the metabolic '70 h change' [40,41], they survive and develop into small adult flies. We therefore subjected larvae expressing *hFOXO3a* or *dFOXO* (under *GMR* control) to either protein starvation (sugar as the only energy source) or complete starvation, starting 80-90 h AEL, and analyzed the effect on the adult's eyes. Both phenotypes (Figure 2k,n) were progressively exacerbated by protein starvation (Figure 2l,o) and complete starvation (Figure 2m), the latter condition being accompanied by early adult or larval lethality, in the case of *hFOXO3a* or *dFOXO*, respectively. The resulting phenotypes are due to the *FOXO* transgenes, as wild-type control flies that have been starved during development display only a body-size reduction while maintaining normal proportions and normal eye structure.

The *dFOXO* overexpression phenotype (Figure 2i,j) does not appear to be caused by the activation of any of the known cell-death pathways. Expression of the caspase inhibitors *p35* or *DIAP1*, or of *p21*, an inhibitor of p53-induced apoptosis [42], and loss of *eiger*, which encodes the *Drosophila* homolog of tumor necrosis factor (TNF) [43], did not suppress the eye phenotype (data not shown). In agreement with our results, it was observed in a parallel study that the *GMR-dFOXO* overexpression phenotype is insensitive to caspase inhibitors, and is not accompanied by increased acridine-orange-detectable apoptosis in the imaginal disc [44]. It therefore remains unclear whether high levels of nuclear dFOXO induce a specific caspase-independent cell-death program or whether nuclear accumulation of overexpressed dFOXO leads to secondary necrosis in a rather nonspecific fashion. Furthermore, the necrotic eye phenotype does not reflect the phenotype observed following a complete block in insulin signaling. Loss-of-function mutations in insulin-signaling components reduce cell size and cell number but do not increase cell death in larval tissues [45,46]. In summary, our overexpression experiments are consistent with a model in which, under normal conditions, excess FOXO transcription factor is phosphorylated by dPKB and kept inactive in the cytoplasm. Under conditions of reduced insulin-signaling activity or nutrient deprivation, dFOXO or hFOXO3a protein translocates to the nucleus and induces growth arrest and necrosis.

dFOXO loss-of-function mutants are viable, have no overgrowth phenotype and are hypersensitive to oxidative stress

Although the overexpression experiments described above did not reveal the physiological function of dFOXO, they

provided the entry point for isolation of loss-of-function mutations. We made use of the *EP35-147* element, which permits the generation of the necrotic eye phenotype (Figure 2j) by driving expression of endogenous *dFOXO* in the presence of Dp110DN. We mutagenized homozygous *EP* males, mated them to homozygous *GMR-Gal4 UAS-Dp110DN* females and then screened the F_1 generation for reversion of the strong gain-of-function phenotype and its associated semilethality. Several loss-of-function alleles of *dFOXO* were isolated and molecularly characterized. Two such revertants are shown in Figure 3c *($dFOXO^{21}$)* and Figure 3d *($dFOXO^{25}$)*. In $dFOXO^{21}$ and $dFOXO^{25}$, the codons for W95 and W124 within the forkhead domain are mutated to stop codons, respectively (Figure 1c), so they are assumed to be null alleles of *dFOXO*. We performed the subsequent phenotypic and epistasis analyses with these two lines.

Because FOXO transcription factors have been proposed to be the primary effectors of insulin signaling, on the basis of epistasis of *daf-16* over *daf-2* in *C. elegans*, it seemed reasonable to expect an overgrowth phenotype in $dFOXO^{-/-}$ flies as is observed in *dPTEN* loss-of-function mutants. To our surprise, *dFOXO* loss-of-function mutants are homozygous-viable and display no obvious phenotype under normal culturing conditions (Figure 3h). Thus, *dFOXO* is not essential for development. Only close inspection of the *dFOXO* mutants revealed that their wing size is significantly reduced (Figure 4i). But cellular and organismal growth are unaffected by *dFOXO* mutations. To assess whether *dFOXO*-mutant tissue grows to a different size than wild-type tissue, we recombined the $dFOXO^{21}$ and $dFOXO^{25}$ alleles onto the *FRT82* chromosome and induced genetic mosaic flies with the *ey-Flp/FRT* system [47]. When the eye and head capsule were composed almost exclusively of $dFOXO^{-/-}$ tissue (w^--marked in Figure 3e,f, on the right), no head-size difference was observed compared to the control fly with a head homozygous for the *FRT82* chromosome without the *dFOXO* mutation (Figure 3e,f, left). This is consistent with experience from extensive genetic screens for recessive growth mutations carried out in our lab. An *ey-Flp*-screen on the right arm of chromosome 3 did not reveal any mutations in *dFOXO* based on an altered head-size phenotype (H.S. and E.H., unpublished observations).

We next asked whether cell size, like organ size, was not affected by the loss of dFOXO. For this purpose, we used a heat shock-inducible *Flp* construct to generate clones of homozygous $dFOXO^{-/-}$ photoreceptor cells and wild-type cells within one adult eye (Figure 3g). The cells lacking dFOXO are marked by the absence of pigment granules. Consistent with the absence of a 'bighead' phenotype, $dFOXO^{-/-}$ cells and wild-type cells have the same size. Similarly, no significant difference in the body weight of mutant and control flies was observed (Figure 3h). In contrast, flies with a viable heteroallelic combination of *dPTEN* loss-of-function alleles are significantly bigger than wild-type flies [48]. Taken together, these results argue that with the exception of the slight wing-size reduction, dFOXO is not required to control cellular, tissue, or organismal growth in a wild-type background.

A critical role has been reported for mammalian and *C. elegans* FOXO proteins in resistance against various cellular stresses, in particular oxidative stress [16,17,49], DNA damage [15] and cytokine withdrawal [50]. We tested the stress resistance of adult *dFOXO* mutant flies by measuring survival time following different challenges. Among starvation on water, oxidative-stress challenge, bacterial infection, heat shock, and heavy-metal stress, the only condition for which hypersensitivity was observed is oxidative stress. When placed on hydrogen-peroxide-containing food, *dFOXO* mutant flies display a significantly reduced survival time compared to control flies (Figure 3i). A very similar effect is elicited by paraquat feeding. These observations are consistent with the paraquat hypersensitivity of *daf-16* mutants in *C. elegans* [51], suggesting that a role for FOXO proteins in protecting against oxidative stress is conserved across species.

Figure 3 *(see figure on the next page)*
Null *dFOXO* mutants are viable, have no overgrowth phenotype and are hypersensitive to oxidative stress. **(a)** *Dp110DN* expressing control fly. **(b)** *EP*-driven coexpression of *dFOXO* elicits a necrotic eye phenotype. **(c,d)** EMS-induced mutations in *dFOXO* lead to a reversion of the overexpression phenotype. **(e,f)** Selective removal of dFOXO from the head (right) does not lead to an organ-size alteration compared to a control fly (left). **(g)** w^--marked dFOXO-deficient photoreceptor cells are the same size as wild-type cells. **(h)** In contrast to *dPTEN*, *dFOXO* null mutants have no organismal growth phenotype. For each genotype, the left bar indicates the body weight of females and the right bar the weight of males. Values are shown ± standard deviation (SD). **(i)** *dFOXO* mutants are hypersensitive to oxidative stress. The graph shows a survival curve of male adult flies on PBS/sucrose gel containing 5% hydrogen peroxide. The observed hypersensitivity is more pronounced in males, but is also observed in females (not shown). The increased resistance of homozygous *EP-dFOXO* flies might be caused by low basal *dFOXO* overexpression from the *EP* element, which occurs due to leakiness of *UAS* enhancers in the absence of Gal4. Control flies placed on PBS/sucrose without oxidant survived during the time window shown. Genotypes are: (a) *y w; GMR-Gal4 UAS-Dp110DN/+*; (b) *y w; GMR-Gal4 UAS-Dp110DN/+; EP-dFOXO/+*; (c) *y w; GMR-Gal4 UAS-Dp110DN/+; EP-dFOXO^{21}/+*; (d) *y w; GMR-Gal4 UAS-Dp110DN/+; EP-dFOXO^{25}/+*; (e,f) *y w ey-flp/y w; FRT82/FRT82 cl3R3 w$^+$* (left); *y w ey-flp/y w; FRT82 EP-dFOXO^{21}/FRT82 cl3R3 w$^+$* (right); (g) *y w hs-flp/y w; FRT82 EP-dFOXO^{21}/FRT82 w$^+$*.

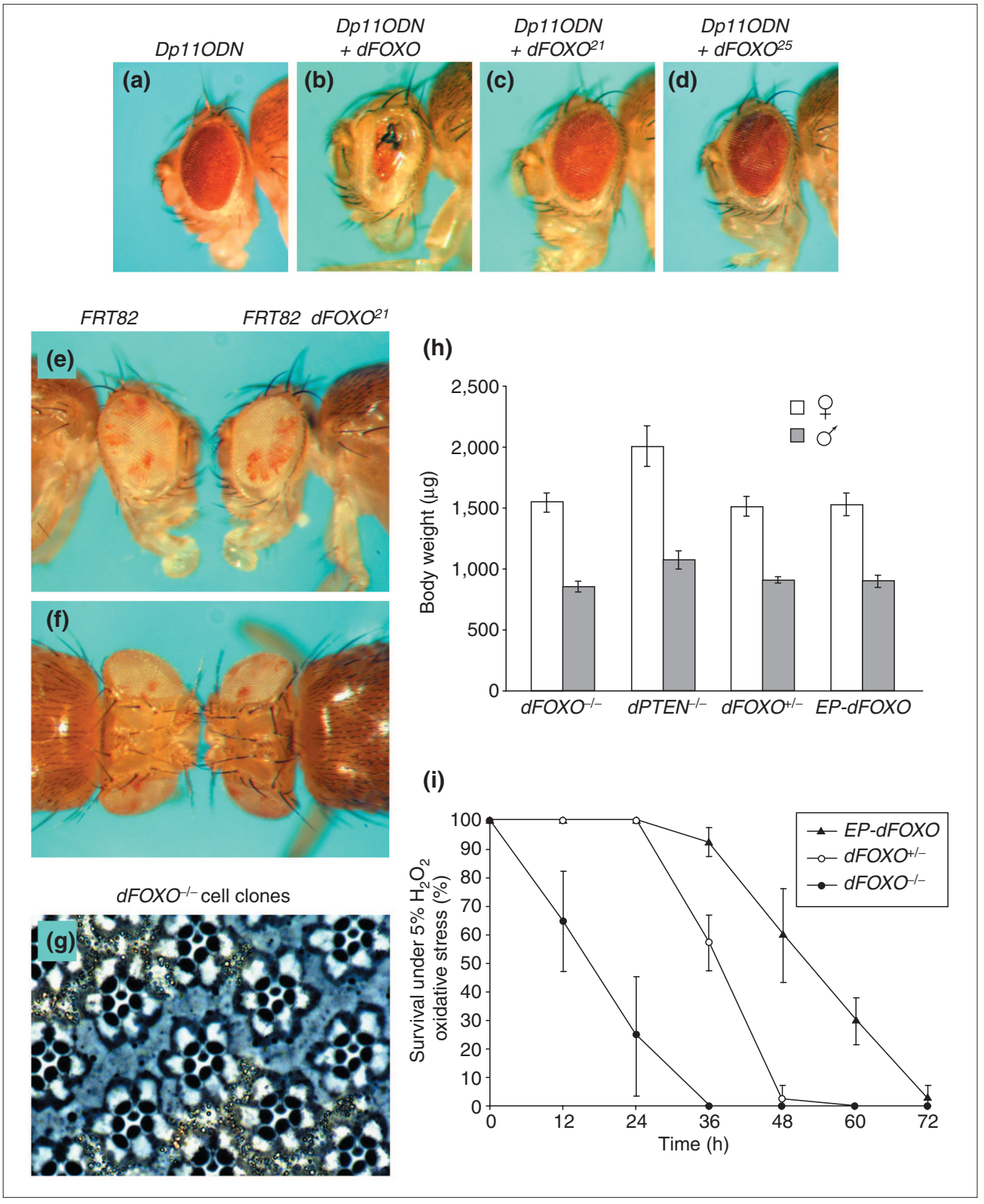


Figure 3 *(see legend on the previous page)*

The growth-deficient phenotypes of *DInr*, *chico*, *Dp110* and *dPKB* mutants are significantly suppressed by loss of dFOXO

We performed genetic epistasis experiments to examine whether the growth phenotypes of DInr-signaling mutants are dependent on dFOXO function. For this purpose, we either generated double-mutant flies or investigated the double-mutant effect only in the head using the *ey-Flp/FRT* system. In contrast to the absence of a growth phenotype in single *dFOXO* mutant flies, lack of dFOXO significantly suppresses the growth-deficient phenotype observed in flies mutant for the insulin receptor substrate (IRS) homolog *chico* (Figure 4). Flies mutant for *chico* are smaller because they have fewer and smaller cells [45]. Loss of one *dFOXO* copy dominantly suppresses the cell-number reduction in *chico* mutant flies without affecting cell size. The suppression is more pronounced when both copies of *dFOXO* are removed in a *chico* mutant background. In this situation, the *chico* small body-size phenotype is partially suppressed. Homozygous *chico-dFOXO* double-mutant flies have more, and even slightly smaller, cells than homozygous *chico* single mutants. It seems that removal of *dFOXO* accelerates the cell cycle at the expense of cell size in a *chico* background.

We next asked whether *dFOXO* interacts with other components of the *Drosophila* insulin-signaling pathway. The *ey-Flp/FRT* system was used to generate heterozygous insulin-signaling mutant flies with heads homozygous for each mutation. Removal of *DInr*, *Dp110* or *dPKB* leads to a characteristic 'pinhead' phenotype, which is substantially suppressed by the presence of a *dFOXO* loss-of-function allele on the same *FRT* chromosome as the insulin-signaling mutation. In all three cases, we observed a partial rather than a complete rescue of the tissue growth repression, consistent with the finding that *dFOXO* mutations affect only the cell-number aspect of the *chico* phenotype. Surprisingly, loss of dFOXO dramatically delays lethality in *dPKB* mutants. Complete loss of dPKB leads to larval lethality in the early third instar, but homozygous *dPKB-dFOXO* double mutants are able to develop into pharate adults of reduced size, most of which fail to eclose (Figure 5l). The lethality associated with the complete loss of dPKB is therefore largely due to hyperactivation of dFOXO.

We also observed that *dFOXO* interacts with the tumor suppressors *dTSC1* and *dPTEN*. Tissue-specific removal of either gene from the head leads to a bighead phenotype (Figure 5h,j). The $dTSC1^{-/-}$ bighead phenotype is enhanced by loss of dFOXO (Figure 5i). This observation is consistent with the recently reported negative feedback loop between dS6K and dPKB. Mutant *dTSC1* larvae have elevated levels of dS6K activity, which in turn downregulates dPKB activity [31]. This reduction in dPKB activity probably leads to enhanced activation of dFOXO, which in turn partially mitigates the overgrowth phenotype by slowing down proliferation. The *dTSC1* phenotype can therefore be enhanced by loss of the inhibitory function of dFOXO. Unexpectedly, the $dPTEN^{-/-}$ bighead phenotype was slightly suppressed by *dFOXO* mutations (Figure 5k). From the current model, it would be expected that in a *dPTEN* mutant dPKB activity is high and dFOXO is to a large extent inactive in the cytoplasm. Thus, removal of dFOXO function should have no effect on the *dPTEN* phenotype. At present, we can only speculate about possible explanations for this observation. In a parallel study, it has been shown that dFOXO can induce transcription of *DInr* [52]. It may be that in a *dPTEN*-mutant background dFOXO activates DInr expression in a negative-feedback loop. In this model, concomitant loss of dFOXO would alleviate the *dPTEN* overgrowth phenotype by lowering DInr levels. Another possible explanation is that dFOXO has additional functions when localized to the cytoplasm or during its nuclear export, such as interacting with other proteins. Loss of dFOXO might affect the function of interaction partners that have a role in dPTEN signaling.

In summary, our epistasis analysis provides strong genetic evidence that dFOXO is required to mediate the organismal growth arrest that is elicited in insulin-signaling mutants.

dFOXO upregulates transcription of the *d4E-BP* gene

We have shown previously that *Drosophila* embryonic Kc167 cells respond to insulin stimulation with upregulated activities of dPKB and dS6K [53,54]. We performed mRNA profiling experiments using the Affymetrix GeneChip system to measure on a genome-wide scale the transcriptional changes induced by insulin in these cells. On the basis of the currently held model that FOXO transcription factors are transcriptional activators that are negatively regulated by insulin, we expected potential dFOXO target genes to be repressed in Kc167 cells upon insulin stimulation. Figure 6a shows a selection of dFOXO target gene candidates that are transcriptionally downregulated by a factor of two or more upon insulin stimulation and whose promoter regions contain one or more conserved forkhead-response elements (FHREs) with the consensus sequence (G/A)TAAACAA [55]. Three of these candidate gene products are each involved in one of two biological processes known to be negatively regulated by insulin, namely gluconeogenesis (PEPCK) and lipid catabolism (CPTI and long-chain-fatty-acid-CoA-ligase). The remaining candidates are involved in stress responses (cytochrome P450 enzymes), DNA repair (DNA polymerase iota), transcription and translation control (d4E-BP and CDK8), and cell-cycle control (centaurin gamma and CG3799). Several of the insulin-repressed genes have been reported to be transcriptionally induced in

Drosophila larvae under conditions of complete starvation (d4E-BP and PEPCK) or sugar-only diet (CPTI and long-chain-fatty-acid-CoA-ligase) [41,56].

We chose d4E-BP for further investigation, because it has previously been reported to be insulin-regulated at the level of protein phosphorylation, but not at the level of gene expression

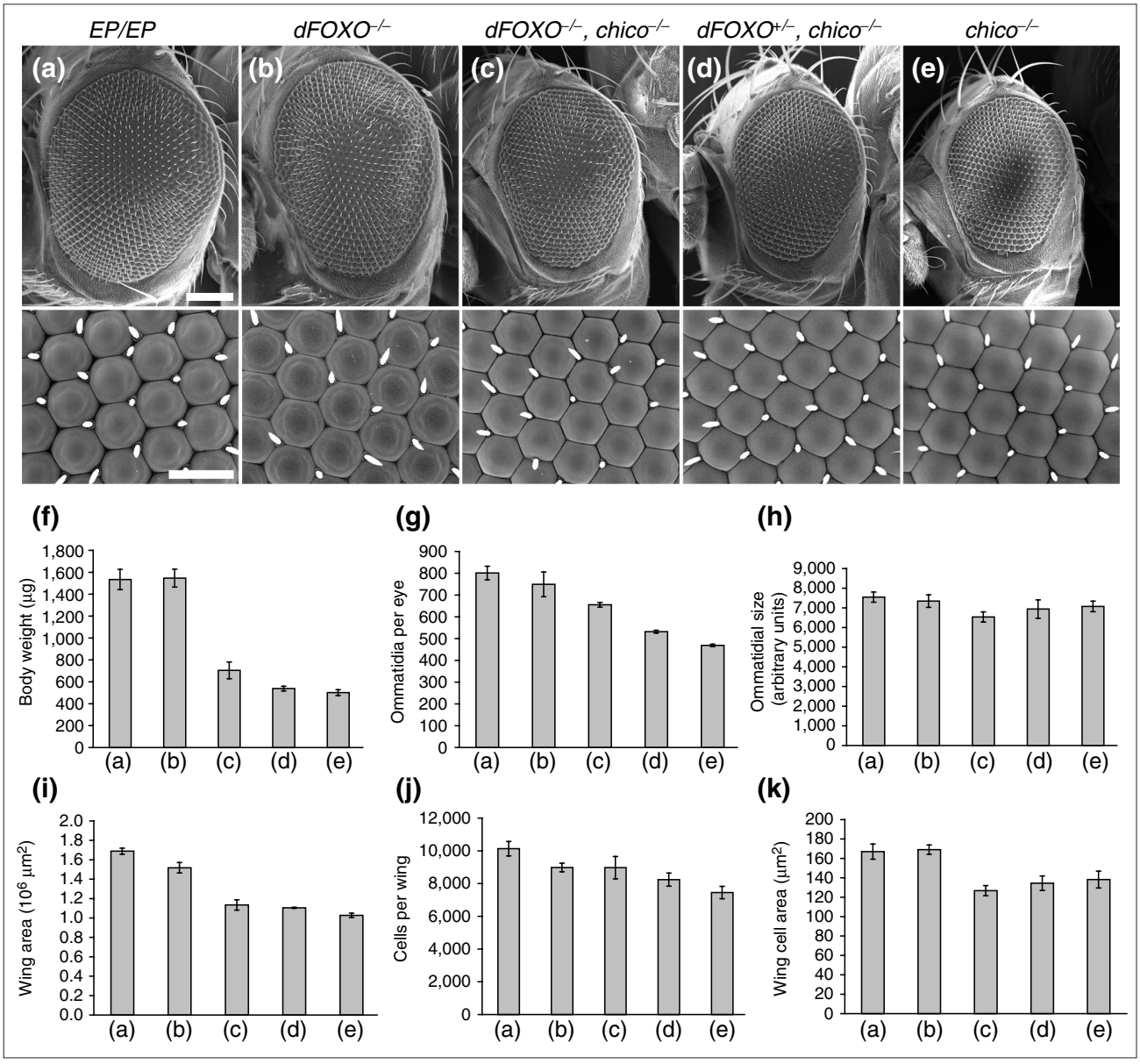


Figure 4

Loss of dFOXO suppresses the cell-number reduction in *chico* mutants. **(a-e)** Partial rescue of the *chico* phenotype by mutations in *dFOXO*. Bar sizes are 100 μm (low magnification) and 20 μm (high magnification). Each graph displays the variation of a single parameter between the five genotypes shown in (a–e): **(f)** body weight, **(g)** cell number in the eye, **(h)** cell size in the eye, **(i)** wing area, **(j)** cell number in the wing, and **(k)** cell size in the wing. (f) *dFOXO*$^{-/-}$ partially suppresses the low-body-weight phenotype of *chico*$^{-/-}$. The suppression is less pronounced in the wing (i), because *dFOXO*-null mutants have significantly smaller wings than control flies, although their body weight is the same. In a *chico*$^{-/-}$ background, loss of dFOXO leads to increased cell numbers in the eye (g) and in the wing (j) compared to the *chico* single mutant. Although organ and tissue size is increased, cell size significantly decreases in the *chico-dFOXO* double mutant both in the eye (h) and in the wing (k). It seems that loss of dFOXO in a *chico*$^{-/-}$ background leads to increased proliferation rates. All values are shown ± SD. Genotypes are: (a) *y w;; EP-dFOXO/EP-dFOXO*; (b) *y w;; EP-dFOXO*^{21}*/EP-dFOXO*^{25}; (c) *y w; chico*^{1}*/chico*^{2}*; EP-dFOXO*^{21}/+; (d) *y w; chico*^{1}*/chico*^{2}*; EP-dFOXO*^{21}*/ EP-dFOXO*^{25}; (e) *y w; chico*^{1}*/chico*^{2}.

[57]. The *d4E-BP* gene encodes a translational repressor and was initially identified as the immune-compromised *Thor* mutant in a genetic screen for genes involved in the innate immune response to bacterial infection [58,59]. Figure 6b shows the presence of several FHREs in the genomic region around the *d4E-BP* locus. The d4E-BP protein is negatively

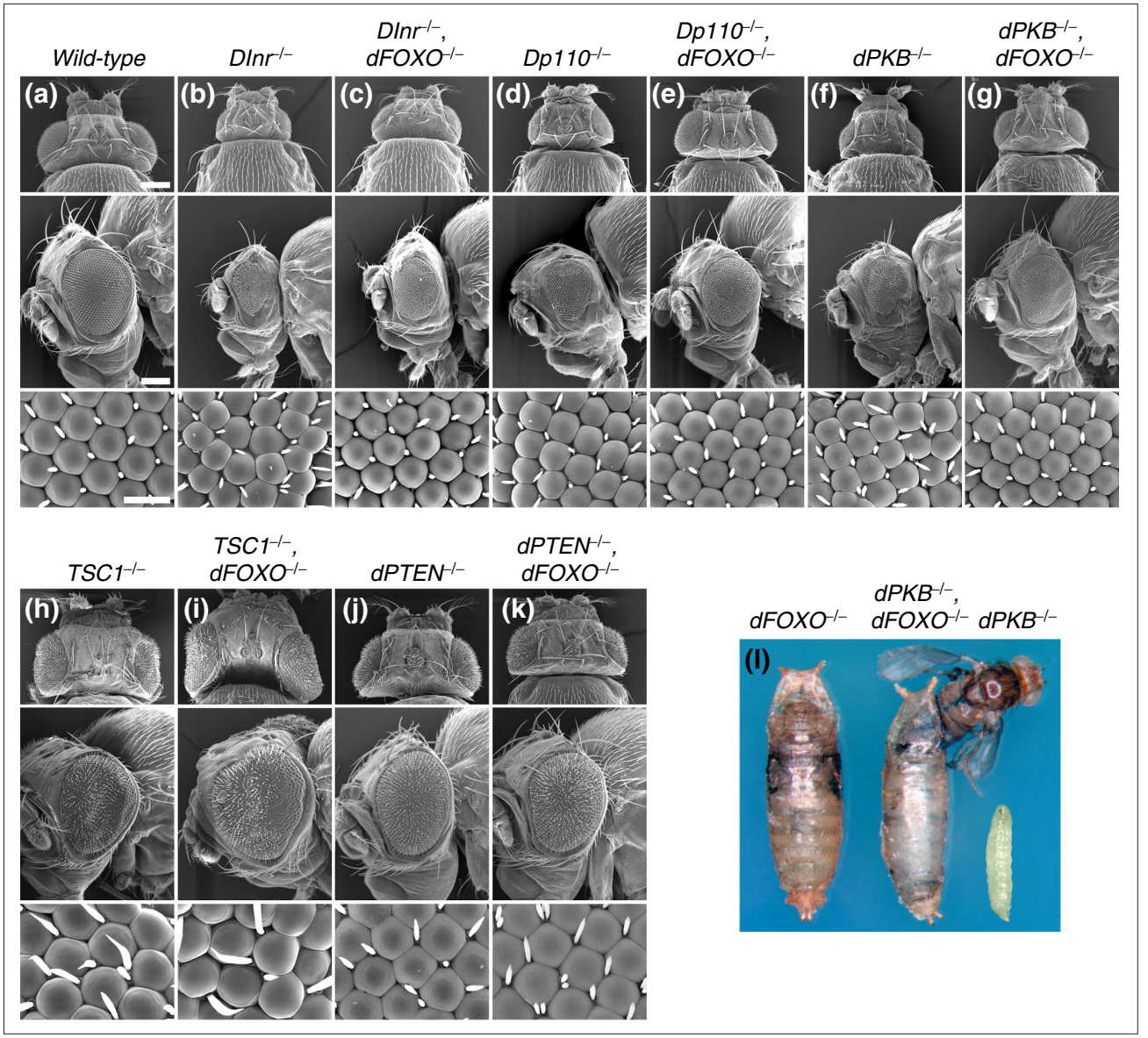
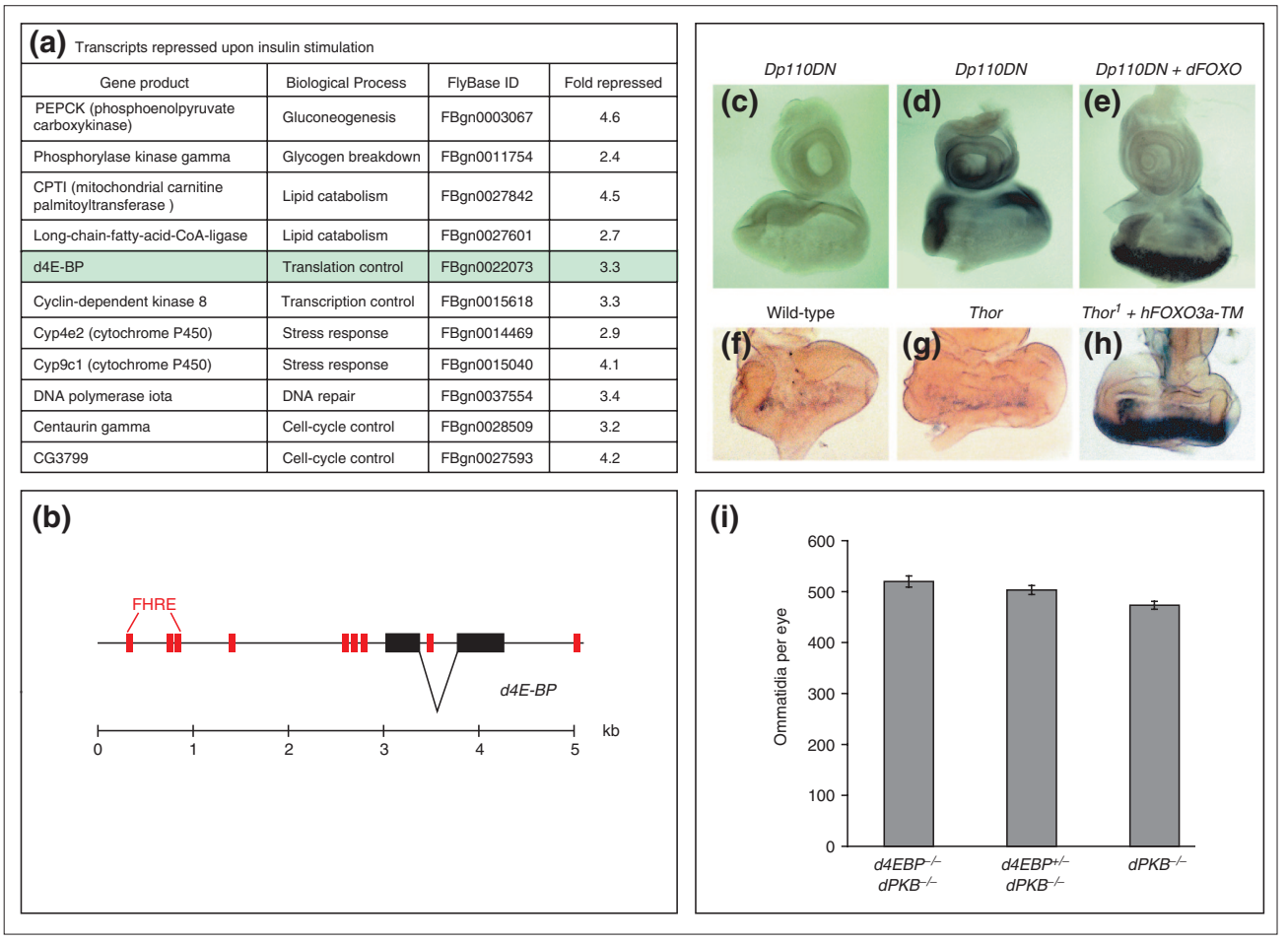


Figure 5
Growth-deficient phenotypes of *DInr*, *Dp110* and *dPKB* mutants are suppressed by loss of dFOXO. **(a)** Control fly. **(b)** Selective removal of DInr from the head leads to a pinhead phenotype, which is partially suppressed by the loss of dFOXO **(c)**. The same suppression is observed in *Dp110*-, and *dPKB*-pinheads **(d-g)**. The *TSC1*$^{-/-}$ bighead phenotype **(h)** is enhanced by mutations in *dFOXO* **(i)**, but the *dPTEN*$^{-/-}$ bighead **(j)** is slightly suppressed **(k)**. **(l)** Living without PKB. In contrast to the larval lethality of *dPKB* null mutants, *dPKB-dFOXO* double mutants develop into small pharate adults, most of which fail to eclose. Bar sizes are 200 μm (low magnification) and 20 μm (high magnification). Genotypes are: (a) *y w ey-flp/y w; FRT82/FRT82 cl3R3 w*$^{+}$; (b) *y w ey-flp/y w; FRT82 DInr*^{304}*/FRT82 cl3R3 w*$^{+}$; (c) *y w ey-flp/y w; FRT82 DInr*^{304} *EP-dFOXO*^{25}*/FRT82 cl3R3 w*$^{+}$; (d) *y w ey-flp/y w; FRT82 Dp110*^{5W3}*/FRT82 cl3R3 w*$^{+}$; (e) *y w ey-flp/y w; FRT82 Dp110*^{5W3} *EP-dFOXO*^{25}*/FRT82 cl3R3 w*$^{+}$; (f) *y w ey-flp/y w; FRT82 dPKB*^{1}*/FRT82 cl3R3 w*$^{+}$; (g) *y w ey-flp/y w; FRT82 dPKB*^{1} *EP-dFOXO*^{25}*/FRT82 cl3R3 w*$^{+}$; (h) *y w ey-flp/y w; FRT82 dTSC1*^{Q87X}*/FRT82 cl3R3 w*$^{+}$; (i) *y w ey-flp/y w; FRT82 dTSC1*^{Q87X} *EP-dFOXO*^{25}*/FRT82 cl3R3 w*$^{+}$; (j) *y w ey-flp/y w; FRT40 dPTEN*$^{117-4}$*/FRT40 cl2L3 w*$^{+}$; (k) *y w ey-flp/y w; FRT40 dPTEN*$^{117-4}$*/FRT40 cl2L3 w*$^{+}$; *FRT82 EP-dFOXO*^{25}*/FRT82 cl3R3 w*$^{+}$; (l) *y w;; EP-dFOXO*^{21}*/EPdFOXO*^{25} (left), *y w;; dPKB*^{1} *EP-dFOXO*^{21}*/dPKB*^{1} *EP-dFOXO*^{25} (middle), *dPKB*^{1}*/dPKB*^{1} (right).



(a) Transcripts repressed upon insulin stimulation

Gene product	Biological Process	FlyBase ID	Fold repressed
PEPCK (phosphoenolpyruvate carboxykinase)	Gluconeogenesis	FBgn0003067	4.6
Phosphorylase kinase gamma	Glycogen breakdown	FBgn0011754	2.4
CPTI (mitochondrial carnitine palmitoyltransferase)	Lipid catabolism	FBgn0027842	4.5
Long-chain-fatty-acid-CoA-ligase	Lipid catabolism	FBgn0027601	2.7
d4E-BP	Translation control	FBgn0022073	3.3
Cyclin-dependent kinase 8	Transcription control	FBgn0015618	3.3
Cyp4e2 (cytochrome P450)	Stress response	FBgn0014469	2.9
Cyp9c1 (cytochrome P450)	Stress response	FBgn0015040	4.1
DNA polymerase iota	DNA repair	FBgn0037554	3.4
Centaurin gamma	Cell-cycle control	FBgn0028509	3.2
CG3799	Cell-cycle control	FBgn0027593	4.2

Figure 6

dFOXO regulates transcription of the *d4E-BP* gene. **(a)** A selection of microarray-identified genes that are transcriptionally downregulated after 2 h of insulin stimulation in Kc167 cells and contain forkhead response elements (FHREs) in their genomic upstream or intronic sequences. **(b)** FHREs (red) at the *d4E-BP* locus; black boxes are exons. **(c,d)** Overexpression of *Dp110DN* alone does not induce transcription of *d4E-BP* in imaginal discs, but **(e)** coexpression of *dFOXO* strongly upregulates the gene. **(f-h)** Expression of human *FOXO3a-TM* induces expression of the *d4E-BP* enhancer trap line *Thor*^{1}. **(i)** *d4E-BP* and *dPKB* interact genetically. The *Thor*^{1} mutation increases the ommatidial number in *dPKB*-mutants by 9% without affecting cell size. Values are shown ± SD. Genotypes are: (c) *y w; GMR-Gal4 UAS-Dp110DN/+*; (d) *y w; GMR-Gal4 UAS-Dp110DN/+*; (e) *y w; GMR-Gal4 UAS-Dp110DN/+; EP-dFOXO/+*; (f) *y w*; (g) *y w; Thor*^{1}*/+*; (h) *y w; Thor*^{1}*/GMR-Gal4; UAS-hFOXO3a-TM/+*; (i) from right to left: *y w;; dPKB*^{3}*/dPKB*^{1}, *y w; Thor*^{1}*/+; dPKB*^{3}*/dPKB*^{1}, *y w; Thor*^{1}*/Thor*^{1}*; dPKB*^{3}*/dPKB*^{1}.

regulated by insulin through LY294002- and rapamycin-sensitive phosphorylation [57], suggesting involvement of the Dp110 and dTOR signaling pathways. Phosphorylation of d4E-BP leads to the dissociation of d4E-BP from its binding partner, the translation initiation factor deIF4E, which then participates in the formation of a functional initiation complex. Positive transcriptional regulation of d4E-BP by dFOXO, which corresponds to negative transcriptional regulation by insulin, would be a complementary mechanism of regulation.

We then investigated whether overexpression of endogenous *dFOXO* could induce transcriptional upregulation of the *d4E-BP* gene. On the basis of our overexpression results, we chose the *Dp110DN-dFOXO* coexpression to efficiently activate dFOXO. Eye imaginal discs from *Dp110DN*-expressing third instar larvae display a low level of basal *d4E-BP* transcription throughout the disc, which is not induced by the driver construct alone (Figure 6d). Coexpression of *dFOXO* elicited a dramatic upregulation of *d4E-BP* transcription posterior to the morphogenetic furrow (Figure 6e). Consistent with this observation, we were able to induce expression of the *d4E-BP* enhancer trap line *Thor*^{1} with human *FOXO3a-TM* (Figure 6f-h). It remained unclear, however, whether regulation of *d4E-BP* expression by dFOXO is of physiological relevance.

It has been previously reported that overexpression of *d4E-BP* partially suppresses the *dPKB* overexpression phenotype [57], but as ectopic expression experiments have to be interpreted with some caution, we assessed whether loss of d4E-BP function suppresses the cell-number reduction in insulin-signaling mutants as does loss of dFOXO function. We generated double-mutant flies for *dPKB* and *d4E-BP* and observed that the *Thor*^{1} mutation slightly but significantly suppressed the reduced cell-number phenotype in a dose-dependent manner. The *Thor*^{1} mutation itself had no effect on ommatidial number compared to wild-type flies (data not shown), so we can rule out additive effects of *d4E-BP* and *dPKB*. These observations strongly argue that under conditions of reduced insulin-signaling activity the dFOXO-dependent reduction in cell number is in part mediated by the transcriptional upregulation of its target *d4E-BP*. Microarray studies in both mammalian [23] and *Drosophila* [52] cells imply that FOXO transcription factors exert their physiological functions by modulating expression of large sets of target genes.

Discussion

Forkhead transcription factors of the FOXO subfamily mediate insulin-regulated gene expression in *C. elegans* and mammals. In this study, we provide genetic evidence that the *Drosophila* FOXO/DAF-16 homolog dFOXO is an important downstream effector of *Drosophila* insulin signaling and a regulator of stress resistance.

dFOXO is a critical target of dPKB but mediates only part of its function

Genetic studies in *C. elegans* and *Drosophila* have led to two models regarding the output of the insulin pathway. First, the complete epistasis of *daf-16* over the insulin pathway mutants *daf-2*, *age-1*, *akt-1* and *akt-2* suggests that the primary function of PKB is to inactivate FOXO transcription factors [60]. Second, it has been proposed that the TSC tumor suppressor complex is the major target of PKB [61,62] in the regulation of cell growth in *Drosophila*. Our analysis of *Drosophila* FOXO indicates that it is indeed a critical PKB target, but that it mediates only one aspect of PKB function. Several lines of evidence support this model. Firstly, the effects of ectopic overexpression of dFOXO and hFOXO3a in the developing *Drosophila* eye are altered by Dp110 and dPKB signaling as well as by nutrient levels. Under conditions of lowered insulin signaling, the phenotypes resulting from expression of *dFOXO* and *hFOXO3a* were dramatically enhanced. This situation was mimicked by expressing a dPKB-insensitive phosphorylation mutant, suggesting that endogenous dPKB signaling is required to mitigate the effects of ectopically expressed dFOXO and hFOXO3a. Secondly, the physiological relevance of dFOXO in dPKB signaling is most vividly demonstrated by our observation that the larval lethality associated with the complete loss of dPKB is rescued by *dFOXO* mutations to the extent that some flies develop to pharate adults. The lethality associated with loss of dPKB function is therefore to a large extent due to the hyperactivation of dFOXO. Thirdly, loss of dFOXO function suppresses the effects of insulin-signaling mutations only partially; dFOXO mediated a reduction in cell number but not in cell size in response to reduced insulin signaling.

dFOXO controls the reduction in cell number in body-size mutants

Genetic analysis of the control of body size in *Drosophila* has revealed two classes of mutations. Flies carrying mutations in *chico* or viable allelic combinations of *DInr*, *Dp110*, and *dPKB* are reduced in body size by up to 50% owing to a reduction in both cell size and cell number. Conversely, flies mutant for *dS6K* exhibit a more moderate reduction in body size, caused almost exclusively by a reduction in cell size [36]. This suggests that the pathways controlling cell number and cell size bifurcate at or below dPKB. Although *dFOXO* single mutants have no obvious size phenotype, loss of *dFOXO* substantially suppresses the cell-number reduction observed in insulin-signaling mutants. It appears that dFOXO mediates the repression of proliferation in flies mutant for *DInr*, *chico*, *Dp110*, and *dPKB* without being required for the reduction in cell size. *Chico-dFOXO* double mutant flies even have slightly smaller cells than *chico* mutants, suggesting that removal of dFOXO permits cell-cycle acceleration under conditions of impaired insulin signaling. The pathway controlling body size in response to insulin therefore bifurcates at the level of dPKB: dPKB controls cell number by inhibiting dFOXO function and dPKB controls cell size, at least under some conditions, by regulating S6K activity by phosphorylation of dTSC2 [29].

The signaling systems controlling cell size and cell number are tightly interconnected. Genetic and biochemical analyses have revealed five different links between the dTSC-dTOR-dS6K pathway and the DInr-dPKB-dFOXO pathway. First, under conditions of unnaturally high insulin-signaling activity (that is, following the oncogenic activation of dPKB) dPKB phosphorylates and inactivates dTSC2, resulting in increased activation of dS6K [29]. Under normal culture conditions this regulation does not seem critical, however, loss of dPKB function does not lower dS6K activity in larval extracts [54]. Second, under physiological conditions, dPDK1 regulates dPKB as well as dS6K [63]. Third, dS6K itself downregulates dPKB activity in a negative feedback loop [31]. Fourth, under severe starvation conditions, nuclear dFOXO presumably activates target genes that reduce cell proliferation. One of these target genes is

d4E-BP, which encodes an inhibitor of translation initiation. When conditions improve, the insulin and TOR signaling pathways can stimulate translation by disrupting the 4E-BP/eIF4E complex via phosphorylation of 4E-BP, and in parallel by repressing FOXO-dependent 4E-BP expression. Fifth, under even more severe starvation or stress conditions, full activation of dFOXO upregulates expression of the insulin receptor itself, thus rendering the cell hypersensitive to low insulin levels (see [52]). These multiple positive and negative interactions ensure a continuous fine adjustment of the growth rate to changing environmental conditions.

Evolutionary conservation of insulin signaling and FOXO function

Genetic dissection of signaling by insulin and its target DAF-16 has been pioneered in *C. elegans* and has helped to unravel the role of this pathway in dauer formation and longevity. Our analysis shows that the same pathway with the homologous nuclear targets operates in flies in the control of cell growth and proliferation, processes that do not involve insulin signaling in worms. Dauer formation and possibly longevity affect the entire organism and do not depend on cell-autonomous functions of the insulin signaling pathway [64]. The cell-growth phenotype in *Drosophila*, however, depends on the cell-autonomous functioning of the insulin-signaling cascade [45]. Insects enter diapause in response to diverse environmental cues (nutrients, day length or temperature) and arrest development or the aging process in a manner similar to dauer formation in worms [65]. Ageing, and possibly diapause, is also under the control of the insulin pathway in *Drosophila* [65,66]. It has recently been shown that heterozygous IGF-1R mutant mice also exhibit a prolonged lifespan [3]. It therefore appears that the function of the insulin pathway, its components, and possibly at least some of its targets, have been conserved throughout evolution.

dFOXO may integrate different forms of cellular stress

The longevity phenotype of IGF-1R-deficient mice is associated with enhanced resistance to oxidative stress [3]. It is likely that this phenomenon is due to hyperactivation of FOXO proteins, as several studies have shown that FOXO transcription factors play a role in the oxidative-stress response in mammalian cells [16,17] as well as in *C. elegans* [49]. Our observation that *dFOXO* mutant flies are hypersensitive to oxidative stress confirms that, in addition to their role in insulin signaling, the role of FOXO proteins in protecting against cellular stress is highly conserved. The mechanism by which dFOXO confers oxidative-stress resistance is not yet known. In our microarray experiment, we identified several genes encoding cytochrome P450 enzymes as dFOXO target gene candidates (Figure 6a). As it has been shown that cytochrome P450 enzymes reduce the toxic effects of paraquat in mice [67], they might partially mediate the protective effect of dFOXO. Furthermore, it remains to be established whether the regulation of dFOXO by insulin is required for dFOXO's protective properties. It is tempting to speculate that distinct stress-induced signaling pathways activate dFOXO under conditions of cellular stress, in addition to the negative input from the insulin cascade, as several stress-induced phosphorylation sites are conserved between hFOXO3a and dFOXO (A Brunet and ME Greenberg, personal communication). This view is supported by our observation that overexpression of a FOXO variant that cannot be inactivated by PKB elicits cell death, a phenotype not observed in larval tissues lacking insulin-signaling components [45]. This result argues that dFOXO induces cellular responses that are independent of insulin.

The emerging model postulates that positive and negative inputs converge on FOXO proteins in response to different environmental conditions, making them central and important integrators controlling cellular (cell-cycle progression) and organismal adaptations (dauer formation, diapause and longevity; see Figure 7). Elucidating the positive inputs that converge on FOXO, by mutating conserved phosphorylation sites in the single *Drosophila* homolog of this class, should help us to better understand dFOXO's integrator function.

Materials and methods

Identification of dFOXO

We searched the *Drosophila* genome [68] using a TBLASTN algorithm for sequences with homology to the DNA-binding domain of human FOXO3a (amino acids 157-251). The resultant matches were further assessed for the presence of consensus PKB phosphorylation sites R-X-R-X-X-S/T [37].

We used a genomic DNA stretch flanking the only identified region fulfilling these criteria to search a collection of *Drosophila* expressed sequence tags [69], which eventually identified two clones (LD05569 and LD18492) containing identical full-length cDNA sequences of 3.7 kb length. The *dFOXO* gene is annotated in FlyBase [70] (FBgn0038197) under the name *foxo*.

Generation of plasmids and transgenic flies

The cDNA clone LD05569 contains the full-length *dFOXO* cDNA within the pBS-SK(+/-) vector (Stratagene [71]). To generate a triple PKB phosphorylation mutant of dFOXO, we used PCR-based site-directed mutagenesis (QuickChange, Stratagene) to introduce the three point mutations T44A, S190A and S259A. Primer sequences are available upon

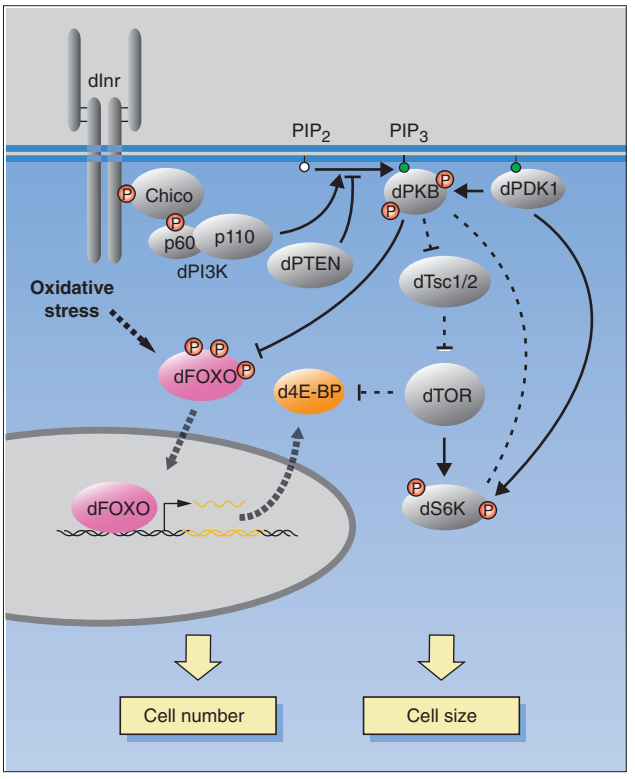


Figure 7
dFOXO may be an integrator of cellular stress. We propose a model in which dFOXO senses different forms of cellular stress (that is, nutrient deprivation or reactive oxygen species) and induces cellular responses, such as proliferation arrest, in part by repressing translation via upregulation of *d4E-BP*. The various signaling proteins shown in the figure are discussed in the text.

request. The mutated sequence was confirmed by double-stranded DNA sequencing. To generate UAS constructs, the cDNA inserts from both wild-type *dFOXO* and triple-mutant *dFOXO* were subcloned from pBS-SK(+/-) into the pUAST transformation vector [72] as *Eco*RI-*Asp*718 fragments. The corresponding UAS constructs containing the cDNA encoding wild-type and triple-mutant hFOXO3a [12] were generated by subcloning the inserts from *pECE-HA-hFOXO3a* and *pECE-HA-hFOXO3a-TM* (generous gifts of Anne Brunet) into pUAST as *Bgl*II-*Xba*I fragments. Fragments were excised from the pECE clones via complete digestion with *Xba*I followed by partial *Bgl*II digestion. All sequences were confirmed by double-stranded DNA sequencing. The four resultant UAS constructs are referred to as *UAS-dFOXO*, *UAS-dFOXO-TM*, *UAS-hFOXO3a* and *UAS-hFOXO3a-TM*. To generate transgenic *Drosophila* lines, P-element-mediated germline transformation was carried out as described previously [73]. Several independent transformant lines were recovered for each construct with the exception of *UAS-dFOXO-TM*, for which we did not obtain a viable transformant line.

EMS reversion mutagenesis

To generate *dFOXO* loss-of-function mutants, homozygous *y w;; EP35-147* males were mutagenized with 27 mM ethyl methanesulfonate (EMS) according to standard procedures [74]. Mutagenized males were mated to homozygous *y w; GMR-Gal4 UAS-Dp110DN* virgins. Roughly 60,000 F_1 progeny were screened for suppression of semilethality and the eye phenotype shown in Figure 3b. F_1 revertants were retested for transmission of the reversion to F_2 and positive candidate lines were then balanced over *TM3 Sb Ser*. To characterize the mutations, the dFOXO open reading frame from each individual mutagenized chromosome was amplified by RT-PCR and sequenced. The cDNA derived from the unmutagenized *EP35-147* chromosome was used as a reference sample to identify mutations. Promising mutations were verified by double peak analysis of PCR fragments amplified from genomic DNA using the Sequencher program (Gene Codes Corporation [75]).

Drosophila strains

The *EP-35-147* line was kindly provided by Konrad Basler, the *GMR-Gal4* driver was a gift from M. Freeman. The *GMR-Gal4, UAS-Dp110DN* line was obtained from Sally Leevers, the *eiger* mutants from Masayuki Miura, and the *Thor*^{1} line from Paul Lasko.

Phenotype analyses

All phenotypes were analyzed in females raised at 25°C unless indicated otherwise. Body weight, cell size and cell number were determined as described previously [5]. The body weight experiment was performed in duplicate, and male and female flies were measured separately (n = 12 for each gender and genotype; the highest and lowest values were excluded from the analysis). Flies were reared under identical, non-crowding conditions and were of identical age (2 d) at the time of the experiment. The sizes of ommatidia and rhabdomeres were quantified with the program NIH Image 1.61. [76].

Clonal analysis

To induce loss-of-function clones, we used the *Flp/FRT* and *ey-Flp* systems to generate mosaic flies by mitotic recombination [47,77]. Overexpression clones were generated as described [63].

In situ hybridizations

In situ hybridizations to eye imaginal discs was performed as described [78,79]. The *d4E-BP* cDNA was PCR-amplified with *Pfu* polymerase from Promega [80] from total double-stranded cDNA derived from adult *y w* flies and cloned

into the pCAP^S vector (PCR blunt-end cloning kit from Roche [81]). Insert orientation was determined by sequencing. Vector-specific PCR primers flanking the multiple cloning site (MCS) and containing either T7 or SP6 RNA polymerase promoters were used to synthesize double-stranded DNA templates for the labeling *in vitro* transcription reaction. The sense probe was transcribed with T7 and the antisense probe with SP6 RNA polymerase.

Cell culture

Drosophila embryonic Kc167 cells were maintained as described elsewhere [53]. Briefly, cells were grown at 25°C in Schneider's *Drosophila* medium (Gibco/Invitrogen [82]) supplemented with 10% heat-inactivated fetal calf serum, FCS. Cells were split and diluted to a density of $1x10^6$ per ml twice a week. For the microarray experiment, cells were grown into the stationary phase for 7 d and then stimulated with 100 nM bovine insulin for 2 h.

Microarray experiment

The microarray experiment was performed at the Functional Genomics Center Zürich (FGCZ) using the Affymetrix GeneChip™ system [83]. Total RNA was extracted from untreated control cells and insulin-treated cells 2 h after stimulation using the RNeasy Mini kit (Qiagen [84]) according to the manufacturer's instructions. From each cell population, three independent samples were taken, processed in parallel and hybridized to three separate microarrays. Synthesis of cDNA and labeled cRNA, array hybridization and scanning were performed according to the standard Affymetrix protocols. The .chp files for the individual scanned microarrays were imported into the Affymetrix Data Mining Tool™ software for data analysis.

Stress treatments

Stress-resistance experiments were performed with 3-day-old adult flies, and males and females were assayed separately. For bacterial infection experiments, adult flies were pricked with a thin needle which had been dipped in a concentrated bacterial culture [85]. Bacterial strains tested were the Gram-negative *Erwinia carotovora carotovora* and the Gram-positive *Micrococcus luteus*. Heat shock was performed by continuous exposure to 37°C. Resistance to heavy metals during development was assayed by rearing flies on food containing either 2.5 mM copper, 6 mM zinc or 200 μM cadmium. For the starvation test, flies were transferred from normal food to empty vials closed with a wet foam stopper. For oxidative-stress challenge, flies were starved in empty vials for 6 h and then transferred to vials containing a gel of phosphate-buffered saline (PBS), 10% sucrose, 0.8% low-melt agarose and the respective oxidative agent (either 5% H_2O_2 or 20 mM paraquat). The oxidant was added to the solution after cooling to 40°C. A control population of flies was placed in vials containing the PBS-sucrose gel without oxidant. Dead flies were counted every 12 h (n = 80 for each gender and genotype). The hydrogen peroxide and paraquat experiments were each done in triplicate. Larval starvation was performed by rearing larvae on normal fly food until 80 h after egg deposition, then floating them in 30% glycerol, washing with water and transfering batches of 30-40 larvae to vials containing a gel of either PBS, 20% sucrose and 0.8% agarose (sugar condition) or PBS-agarose only (complete starvation).

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References

1. Takahashi Y, Kadowaki H, Momomura K, Fukushima Y, Orban T, Okai T, Taketani Y, Akanuma Y, Yazaki Y, Kadowaki T: **A homozygous kinase-defective mutation in the insulin receptor gene in a patient with leprechaunism.** *Diabetologia* 1997, **40:**412-420.
2. Baker J, Liu JP, Robertson EJ, Efstratiadis A: **Role of insulin-like growth factors in embryonic and postnatal growth.** *Cell* 1993, **75:**73-82.
3. Holzenberger M, Dupont J, Ducos B, Leneuve P, Geloen A, Even PC, Cervera P, Le Bouc Y: **IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice.** *Nature* 2003, **421:**182-187.
4. Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G: ***daf*-2, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*.** *Science* 1997, **277:**942-946.
5. Brogiolo W, Stocker H, Ikeya T, Rintelen F, Fernandez R, Hafen E: **An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control.** *Curr Biol* 2001, **11:**213-221.
6. Tatar M, Kopelman A, Epstein D, Tu MP, Yin CM, Garofalo RS: **A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function.** *Science* 2001, **292:**107-110.
7. Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E, Leevers SJ, Partridge L: **Extension of life-span by loss of CHICO, a *Drosophila* insulin receptor substrate protein.** *Science* 2001, **292:**104-106.
8. Cantley LC: **The phosphoinositide 3-kinase pathway.** *Science* 2002, **296:**1655-1657.
9. Maehama T, Dixon JE: **The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate.** *J Biol Chem* 1998, **273:**13375-13378.
10. Stocker H, Andjelkovic M, Oldham S, Laffargue M, Wymann MP, Hemmings BA, Hafen E: **Living with lethal PIP3 levels: viability of flies lacking PTEN restored by a PH domain mutation in Akt/PKB.** *Science* 2002, **295:**2088-2091.

11. Burgering BM, Kops GJ: **Cell cycle and death control: long live Forkheads.** *Trends Biochem Sci* 2002, **27:**352-360.
12. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME: **Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor.** *Cell* 1999, **96:**857-868.
13. Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, Burgering BM: **Direct control of the Forkhead transcription factor AFX by protein kinase B.** *Nature* 1999, **398:**630-634.
14. Brunet A, Park J, Tran H, Hu LS, Hemmings BA, Greenberg ME: **Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a).** *Mol Cell Biol* 2001, **21:**952-965.
15. Tran H, Brunet A, Grenier JM, Datta SR, Fornace AJ, Jr., DiStefano PS, Chiang LW, Greenberg ME: **DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein.** *Science* 2002, **296:**530-534.
16. Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM: **Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress.** *Nature* 2002, **419:**316-321.
17. Furukawa-Hibi Y, Yoshida-Araki K, Ohta T, Ikeda K, Motoyama N: **FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress.** *J Biol Chem* 2002, **277:**26729-26732.
18. Dijkers PF, Medema RH, Lammers JW, Koenderman L, Coffer PJ: **Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1.** *Curr Biol* 2000, **10:**1201-1204.
19. Dijkers PF, Medema RH, Pals C, Banerji L, Thomas NS, Lam EW, Burgering BM, Raaijmakers JA, Lammers JW, Koenderman L, *et al.*: **Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1).** *Mol Cell Biol* 2000, **20:**9138-9148.
20. Alvarez B, Martinez AC, Burgering BM, Carrera AC: **Forkhead transcription factors contribute to execution of the mitotic programme in mammals.** *Nature* 2001, **413:**744-747.
21. Medema RH, Kops GJ, Bos JL, Burgering BM: **AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1.** *Nature* 2000, **404:**782-787.
22. Barthel A, Schmoll D, Kruger KD, Bahrenberg G, Walther R, Roth RA, Joost HG: **Differential regulation of endogenous glucose-6-phosphatase and phosphoenolpyruvate carboxykinase gene expression by the forkhead transcription factor FKHR in H4IIE-hepatoma cells.** *Biochem Biophys Res Commun* 2001, **285:**897-902.
23. Ramaswamy S, Nakamura N, Sansal I, Bergeron L, Sellers WR: **A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR.** *Cancer Cell* 2002, **2:**81-91.
24. Schmidt M, de Mattos SF, van der Horst A, Klompmaker R, Kops GJ, Lam EW, Burgering BM, Medema RH: **Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D.** *Mol Cell Biol* 2002, **22:**7842-7852.
25. Nakae J, Biggs WH, 3rd, Kitamura T, Cavenee WK, Wright CV, Arden KC, Accili D: **Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1.** *Nat Genet* 2002, **32:**245-253.
26. Borkhardt A, Repp R, Haas OA, Leis T, Harbott J, Kreuder J, Hammermann J, Henn T, Lampert F: **Cloning and characterization of AFX, the gene that fuses to MLL in acute leukemias with a t(X;11)(q13;q23).** *Oncogene* 1997, **14:**195-202.
27. Sublett JE, Jeon IS, Shapiro DN: **The alveolar rhabdomyosarcoma PAX3/FKHR fusion protein is a transcriptional activator.** *Oncogene* 1995, **11:**545-552.
28. Inoki K, Li Y, Zhu T, Wu J, Guan KL: **TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling.** *Nat Cell Biol* 2002, **4:**648-657.
29. Potter CJ, Pedraza LG, Xu T: **Akt regulates growth by directly phosphorylating Tsc2.** *Nat Cell Biol* 2002, **4:**658-665.
30. Gao X, Zhang Y, Arrazola P, Hino O, Kobayashi T, Yeung RS, Ru B, Pan D: **Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling.** *Nat Cell Biol* 2002, **4:**699-704.
31. Radimerski T, Montagne J, Hemmings-Mieszczak M, Thomas G: **Lethality of *Drosophila* lacking TSC tumor suppressor function rescued by reducing dS6K signaling.** *Genes Dev* 2002, **16:**2627-2632.
32. Stocker H, Radimerski T, Schindelholz B, Wittwer F, Belawat P, Daram P, Breuer S, Thomas G, Hafen E: **Rheb is an essential regulator of S6K in controlling cell growth in *Drosophila*.** *Nat Cell Biol* 2003, **5:**559-566.
33. Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D: **Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins.** *Nat Cell Biol* 2003, **5:**578-581.
34. Saucedo LJ, Gao X, Chiarelli DA, Li L, Pan D, Edgar BA: **Rheb promotes cell growth as a component of the insulin/TOR signalling network.** *Nat Cell Biol* 2003, **5:**566-571.
35. Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, Kozma SC, Hafen E, Bos JL, Thomas G: **Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2.** *Mol Cell* 2003, **11:**1457-1466.
36. Montagne J, Stewart MJ, Stocker H, Hafen E, Kozma SC, Thomas G: ***Drosophila* S6 kinase: a regulator of cell size.** *Science* 1999, **285:**2126-2129.
37. Alessi DR, Caudwell FB, Andjelkovic M, Hemmings BA, Cohen P: **Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase.** *FEBS Lett* 1996, **399:**333-338.
38. Hay BA, Wassarman DA, Rubin GM: ***Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death.** *Cell* 1995, **83:**1253-1262.
39. Leevers SJ, Weinkove D, MacDougall LK, Hafen E, Waterfield MD: **The *Drosophila* phosphoinositide 3-kinase Dp110 promotes cell growth.** *EMBO J* 1996, **15:**6584-6594.
40. Beadle G, Tatum E, Clancy C: **Food level in relation to rate of development and eye pigmentation in *Drosophila melanogaster*.** *Biol Bull* 1938, **75:**447-462.
41. Zinke I, Schutz CS, Katzenberger JD, Bauer M, Pankratz MJ: **Nutrient control of gene expression in *Drosophila*: microarray analysis of starvation and sugar-dependent response.** *EMBO J* 2002, **21:**6162-6173.
42. Ollmann M, Young LM, Di Como CJ, Karim F, Belvin M, Robertson S, Whittaker K, Demsky M, Fisher WW, Buchman A, *et al.*: ***Drosophila* p53 is a structural and functional homolog of the tumor suppressor p53.** *Cell* 2000, **101:**91-101.
43. Igaki T, Kanda H, Yamamoto-Goto Y, Kanuka H, Kuranaga E, Aigaki T, Miura M: **Eiger, a TNF superfamily ligand that triggers the *Drosophila* JNK pathway.** *EMBO J* 2002, **21:**3009-3018.
44. Kramer JM, Davidge JT, Lockyer JM, Staveley BE: **Expression of Drosophila FOXO regulates growth and can phenocopy starvation.** *BMC Dev Biol* 2003, **3:**5.
45. Bohni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andruss BF, Beckingham K, Hafen E: **Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4.** *Cell* 1999, **97:**865-875.
46. Weinkove D, Neufeld TP, Twardzik T, Waterfield MD, Leevers SJ: **Regulation of imaginal disc cell size, cell number and organ size by *Drosophila* class I(A) phosphoinositide 3-kinase and its adaptor.** *Curr Biol* 1999, **9:**1019-1029.
47. Newsome TP, Asling B, Dickson BJ: **Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics.** *Development* 2000, **127:**851-860.
48. Oldham S, Stocker H, Laffargue M, Wittwer F, Wymann M, Hafen E: **The *Drosophila* insulin/IGF receptor controls growth and size by modulating PtdInsP(3) levels.** *Development* 2002, **129:**4103-4109.
49. Honda Y, Honda S: **The *daf-2* gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*.** *FASEB J* 1999, **13:**1385-1393.
50. Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L, Coffer PJ: **FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity.** *J Cell Biol* 2002, **156:**531-542.

51. Yanase S, Yasuda K, Ishii N: **Adaptive responses to oxidative damage in three mutants of *Caenorhabditis elegans (age-1, mev-1* and *daf-16)* that affect life span.** *Mech Ageing Dev* 2002, **123:**1579-87.
52. Puig O, Marr MT, Ruhf ML, Tjian R: **Control of cell number by *Drosophila* FOXO: downstream and feedback regulation of the insulin receptor pathway.** *Genes Dev,* in press.
53. Radimerski T, Mini T, Schneider U, Wettenhall RE, Thomas G, Jeno P: **Identification of insulin-induced sites of ribosomal protein S6 phosphorylation in *Drosophila melanogaster*.** *Biochemistry* 2000, **39:**5766-5774.
54. Radimerski T, Montagne J, Rintelen F, Stocker H, van der Kaay J, Downes CP, Hafen E, Thomas G: **dS6K-regulated cell growth is dPKB/dPI(3)K-independent, but requires dPDK1.** *Nat Cell Biol* 2002, **4:**251-255.
55. Furuyama T, Nakazawa T, Nakano I, Mori N: **Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues.** *Biochem J* 2000, **349:**629-634.
56. Zinke I, Kirchner C, Chao LC, Tetzlaff MT, Pankratz MJ: **Suppression of food intake and growth by amino acids in *Drosophila*: the role of *pumpless*, a fat body expressed gene with homology to vertebrate glycine cleavage system.** *Development* 1999, **126:**5275-5284.
57. Miron M, Verdu J, Lachance PE, Birnbaum MJ, Lasko PF, Sonenberg N: **The translational inhibitor 4E-BP is an effector of PI(3)K/Akt signalling and cell growth in *Drosophila*.** *Nat Cell Biol* 2001, **3:**596-601.
58. Bernal A, Kimbrell DA: ***Drosophila* Thor participates in host immune defense and connects a translational regulator with innate immunity.** *Proc Natl Acad Sci USA* 2000, **97:**6019-6024.
59. Rodriguez A, Zhou Z, Tang ML, Meller S, Chen J, Bellen H, Kimbrell DA: **Identification of immune system and response genes, and novel mutations causing melanotic tumor formation in *Drosophila* melanogaster.** *Genetics* 1996, **143:**929-940.
60. Paradis S, Ruvkun G: ***Caenorhabditis elegans* Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor.** *Genes Dev* 1998, **12:**2488-2498.
61. Potter CJ, Huang H, Xu T: ***Drosophila* Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size.** *Cell* 2001, **105:**357-368.
62. Gao X, Pan D: **TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth.** *Genes Dev* 2001, **15:**1383-1392.
63. Rintelen F, Stocker H, Thomas G, Hafen E: **PDK1 regulates growth through Akt and S6K in *Drosophila*.** *Proc Natl Acad Sci USA* 2001, **98:**15020-15025.
64. Apfeld J, Kenyon C: **Cell nonautonomy of *C. elegans daf-2* function in the regulation of diapause and life span.** *Cell* 1998, **95:**199-210.
65. Tatar M, Yin C: **Slow aging during insect reproductive diapause: why butterflies, grasshoppers and flies are like worms.** *Exp Gerontol* 2001, **36:**723-738.
66. Partridge L, Gems D: **Mechanisms of ageing: public or private?** *Nat Rev Genet* 2002, **3:**165-175.
67. Shimada H, Furuno H, Hirai K, Koyama J, Ariyama J, Simamura E: **Paraquat detoxicative system in the mouse liver postmitochondrial fraction.** *Arch Biochem Biophys* 2002, **402:**149-157.
68. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, *et al.*: **The genome sequence of *Drosophila melanogaster*.** *Science* 2000, **287:**2185-2195.
69. Rubin GM, Hong L, Brokstein P, Evans-Holm M, Frise E, Stapleton M, Harvey DA: **A *Drosophila* complementary DNA resource.** *Science* 2000, **287:**2222-2224.
70. **FlyBase** [http://flybase.org]
71. **Stratagene** [http://www.stratagene.com]
72. Brand AH, Perrimon N: **Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.** *Development* 1993, **118:**401-415.
73. Basler K, Christen B, Hafen E: **Ligand-independent activation of the sevenless receptor tyrosine kinase changes the fate of cells in the developing *Drosophila* eye.** *Cell* 1991, **64:**1069-1081.
74. Ashburner M: *Drosophila.* Cold Spring Harbor Laboratory Press: Cold Spring Harbor; 1989.
75. **Gene Codes Corporation** [http://www.genecodes.com]
76. **NIH Image** [http://rsb.info.nih.gov/nih-image]
77. Xu T, Rubin GM: **Analysis of genetic mosaics in developing and adult *Drosophila* tissues.** *Development* 1993, **117:**1223-1237.
78. Lehmann R, Tautz D: ***In situ* hybridization to RNA.** *Methods Cell Biol* 1994, **44:**575-598.
79. O'Neill JW, Bier E: **Double-label *in situ* hybridization using biotin and digoxigenin-tagged RNA probes.** *Biotechniques* 1994, **17:**870-875.
80. **Promega** [http://www.promega.com]
81. **Roche** [http://www.roche-applied-science.com]
82. **Invitrogen life technologies** [http://www.invitrogen.com]
83. **Affymetrix** [http://www.affymetrix.com]
84. **Qiagen** [http://www.qiagen.com]
85. Lemaitre B, Reichhart JM, Hoffmann JA: ***Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms.** *Proc Natl Acad Sci USA* 1997, **94:**14614-14619.
86. Weigelt J, Climent I, Dahlman-Wright K, Wikstrom M: **Solution structure of the DNA binding domain of the human forkhead transcription factor AFX (FOXO4).** *Biochemistry* 2001, **40:**5861-5869.

Diet-dependent effects of the *Drosophila* Mnk1/Mnk2 homolog Lk6 on growth via eIF4E

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Diet-Dependent Effects of the *Drosophila* Mnk1/Mnk2 Homolog Lk6 on Growth via eIF4E

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Summary

The control of cellular growth is tightly linked to the regulation of protein synthesis. A key function in translation initiation is fulfilled by the 5′ cap binding eukaryotic initiation factor 4E (eIF4E), and dysregulation of eIF4E is associated with malignant transformation and tumorigenesis [1, 2]. In mammals, the activity of eIF4E is modulated by phosphorylation at Ser209 by mitogen-activated protein kinases (MAPK)-interacting kinases 1 and 2 (Mnk1 and Mnk2) [3–5], which themselves are activated by ERK and p38 MAPK in response to mitogens, cytokines or cellular stress [6]. Whether phosphorylation of eIF4E at Ser209 exerts a positive or inhibitory effect on translation efficiency has remained controversial. Here we provide a genetic characterization of the *Drosophila* homolog of Mnk1/2, Lk6. Lk6 function is dispensable under a high protein diet, consistent with the recent finding that mice lacking both Mnk1 and Mnk2 are not growth-impaired [4]. Interestingly, loss of Lk6 function causes a significant growth reduction when the amino acid content in the diet is reduced. Overexpression of Lk6 also results in growth inhibition in an eIF4E-dependent manner. We propose a model of eIF4E regulation that may reconcile the contradictory findings with regard to the role of phosphorylation by Mnk1/2.

Results and Discussion

Lk6 Overexpression Inhibits Growth through eIF4E

In search of novel growth effectors acting downstream of Protein kinase B (PKB, also called Akt) and 3′-phosphoinositide-dependent kinase-1 (PDK1), we set out to identify genes whose expression is capable of modulating an overgrowth phenotype caused by the cooverexpression of PKB and PDK1. To this end, we performed a UAS (upstream activating sequences)/Gal4-based EP (enhancer/promoter) screen [7, 8] to coexpress random genes together with PKB/PDK1. *EP30.18* strongly suppressed the hyperplastic phenotype (Figure 1A), and plasmid rescue revealed *Lk6* as the gene driven by the EP insertion (henceforth termed *EPLk6*).

Lk6 encodes the single *Drosophila* homolog of Mnk1 and Mnk2. It has previously been described as a microtubule- and centrosome-associated protein [9], and it was also identified in a misexpression screen as a putative negative regulator of Ras/MAPK signaling [10]. However, no physiological role has been assigned to Lk6 in *Drosophila* so far.

EPLk6-mediated Lk6 overexpression not only suppressed the overgrowth phenotype elicited by coexpression of PKB and PDK1, but it also attenuated the eye overgrowth caused by the overproduction of the insulin receptor (Inr) and of the small GTPase Rheb (Ras homolog enriched in brain) as well as an S6 kinase (S6K)-dependent bent-down wing phenotype (see Figure S1 in the Supplemental Data available with this article online; data not shown). Ubiquitous expression of Lk6 (by means of *daGal4*) resulted in smaller flies (weight reduction of 10% in females and 9% in males, respectively; Figure 1B). The reduced wing size (−10%) was brought about by a decrease in cell number (10% decrease in females) but not in cell size (Figure 1C). Likewise, the observed reduction in overall eye size was caused by a diminution in cell number (data not shown).

eIF4E is regulated by binding of eIF4E binding proteins (4E-BPs), which in their hypophosphorylated form inhibit cap-dependent translation, and by direct phosphorylation [11]. The mammalian Lk6 homologs Mnk1 and Mnk2 are the physiological kinases responsible for the phosphorylation of eIF4E at Ser209 [3–5]. Flies lacking eIF4E function die during early larval stages. A genomic *eIF4E* construct containing an amino acid exchange at position 251 (Ser251→Ala, corresponding to Ser209 in mammalian eIF4E) is capable of rescuing *eIF4E* null mutants to adulthood. However, the rescued animals have a reduced viability, develop more slowly, and are smaller than control flies, indicating an important role for Ser251 phosphorylation in assuring normal growth during development [12]. To genetically test whether the effects of Lk6 are mediated by phosphorylation of eIF4E at Ser251, we overexpressed Lk6 with *daGal4* in an *eIF4E*-deficient background provided with an $eIF4E^{Ser251Ala}$ transgene. Strikingly, the effects of Lk6 overexpression were completely abrogated in this genetic context (Figure 1D). Thus, at least under overexpression conditions, eIF4E-Ser251 is required for Lk6 to affect growth.

Lk6 Is Dispensable for Normal Growth and Development

The functional significance of eIF4E-Ser209 phosphorylation is still under debate [13]. It is widely accepted that eIF4E phosphorylation is increased in response to growth factors, hormones, cytokines, and mitogenic stimuli that activate translation [11]. However, different studies demonstrated a negligible role—or even an inhibitory effect—for eIF4E-Ser209 phosphorylation in translation initiation [14, 15]. Moreover, published data concerning the affinity of phosphorylated eIF4E for capped mRNA are also contradictory [16–18]. Thus, it is essential to study the physiological function of eIF4E phosphorylation in a genetically tractable model organism such as *Drosophila*. We generated mutations in *Lk6*

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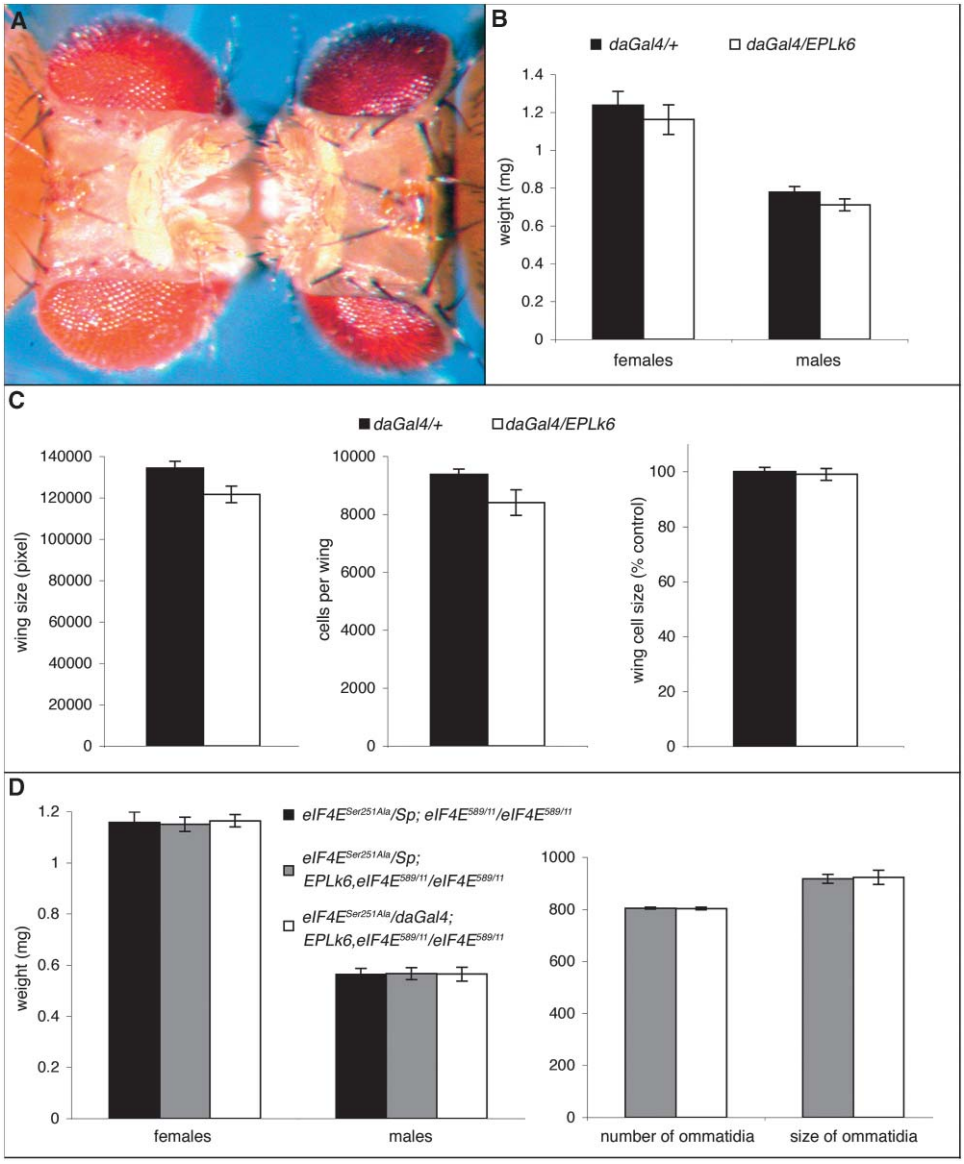

Figure 1. Overexpression of Lk6, the *Drosophila* Mnk1/2 Homolog, Inhibits Growth via Phosphorylation of eIF4E

(A) Overexpression of Lk6 (achieved by *EPLk6*) suppresses a PKB/PDK1-dependent big-eye phenotype. Dorsal view of *Drosophila* adult heads of genotypes *y w;GMR-Gal4 UAS-PKB/+;EPPDK1/+* (left) and *y w;GMR-Gal4 UAS-PKB/+;EPPDK1/EPLk6* (right).

(B) Ubiquitous expression of Lk6 (with *daGal4* as driver line) gives rise to smaller flies. Quantitative analysis shows a weight reduction of 10% in females and 9% in males ($n = 33$, $p < 0.05$; student's t test).

(C) The reduced wing size ($n = 10$, $p < 0.05$; student's t test) is attributable to fewer cells.

(D) Flies overexpressing Lk6 in an *eIF4E* phosphorylation site mutant background (white bars) do not display any weight reduction as compared to control flies (black and gray bars; $n = 19$). Consistently, neither number nor size of ommatidia in the adult eye of females is altered (right; $n = 4$). Thus, Lk6 exerts its effect via phosphorylation of eIF4E at Ser251. Measurements were performed according to Bohni et al. [22]. All error bars represent the standard deviation.

by feeding *EPLk6* males with ethylmethane sulphonate (EMS) and crossing them to *Act5CGal4* driver females. Because the overexpression of Lk6 is lethal at the pupal stage, only flies carrying a lesion in the *Lk6* gene (or in an essential downstream gene) could survive. In this way, we obtained five loss-of-function alleles of *Lk6* (Figure 2A). Four mutations ($Lk6^{15}$, $Lk6^{25}$, $Lk6^{26}$, and $Lk6^{36}$) are located in the region encoding the evolutionarily conserved kinase domain. $Lk6^{38}$ results in a premature stop at amino acid position 474, located C-terminal to the kinase domain (Figure 2A). All mutant combinations gave rise to viable and fertile flies without obvious growth defects (Figure 2B). Clones of *Lk6* mutant cells were induced in imaginal discs during larval stages to investigate whether loss of Lk6 affects growth of mutant cells when juxtaposed to wild-type cells. The growth behavior of marked *Lk6* mutant clones was indistinguishable from their neighboring twin spot clones (Figure 2C). Accordingly, no differences in cell size could be observed in clones of *Lk6* mutant cells in the adult

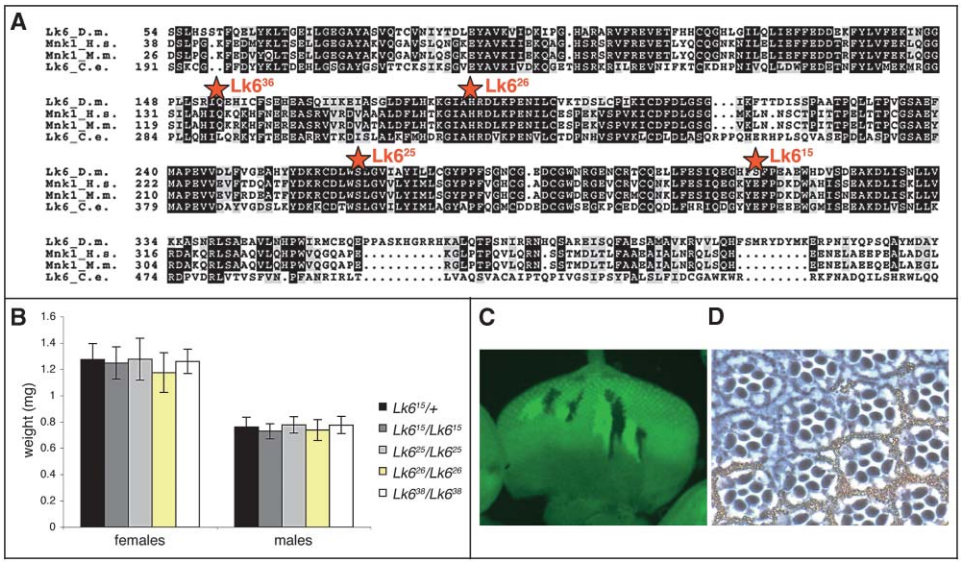

Figure 2. Loss of Lk6 Does Not Lead to Growth Abnormalities under Standard Culture Conditions

(A) Alignment of the highly conserved kinase domain of Lk6 with its human (H.s.), mouse (M.m.), and *C. elegans* (C.e.) homologs (Mnk1 and Mnk2 in mammals; alignment only shown for Mnk1). The red stars indicate the positions of the sequence changes caused by the EMS-induced *Lk6* mutations. $Lk6^{36}$: His to Arg at position 154; $Lk6^{26}$: Gly to Arg at position 186; $Lk6^{25}$: Ser to Leu at position 265; and $Lk6^{15}$: frame shift, at position 360, leading to translation termination after nine additional amino acids (GGRVARCQR). $Lk6^{38}$ results in a truncation at position 474 after the kinase domain.

(B) *Lk6* mutant flies are normal in size. The weight of mutant female and male flies (four different alleles) is not significantly altered compared to control flies.

(C and D) Clonal analyses in imaginal wing discs (C) and in the adult eye (D) do not reveal any growth impairment of *Lk6* mutant cells. Mitotic recombination was induced by a heatshock 48–72 hr after egg deposition. Mutant cells are marked by the absence of GFP and red pigmentation, respectively. Genotypes are *y w hs-Flp;FRT82* $Lk6^{15}$*/FRT82 Ubi-GFP* (C) and *y w hs-Flp;FRT82* $Lk6^{15}$*/FRT82* w^{+} (D).

compound eye (Figure 2D). Thus, Lk6 is dispensable for normal development and growth under standard culture conditions.

Lk6 Is Limiting under Conditions of Reduced Dietary Protein and Oxidative Stress

A comparison with the results of another study on the function of Lk6 ([19], published online along with this paper) revealed surprising differences because these authors observed a significant growth reduction of *Lk6* mutant flies. We reasoned that the difference could originate from unequal culture conditions and tested whether the requirement for Lk6 function depends on the diet. eIF4E is regulated both by phosphorylation and by direct binding to 4E-BPs. The activity of 4E-BPs is intimately linked to nutrient availability because TOR, the kinase that is part of a nutrient-sensing complex [20], phosphorylates 4E-BP under nutrient-rich conditions, leading to the dissociation of 4E-BP from eIF4E [21]. Lk6 function might become limiting when amino acid levels are reduced. *Lk6* mutant as well as control larvae were reared on food with a reduced content in yeast (and therefore limited amino acid supply) to test this hypothesis. At 30% yeast, control flies did not show any weight loss when compared to flies reared at 100% yeast (our standard fly medium). In contrast, *Lk6* mutants were significantly smaller at 30% yeast (weight reduction of 9% in males and 20% in females; Figure 3A; data not shown). The reduced size resulted from a diminution in cell number because cell size was not affected in the adult wings (Figure 3B). Consistently, the number of ommatidia in the compound eye was also reduced (8% fewer ommatidia in females; data not shown). Further reduction of the yeast content (20% yeast) led to an even more pronounced weight loss of the *Lk6* mutants (15% in males, 29% in females), but it also decreased the weight of control flies by up to 6% in males (Figure 3A). Interestingly, the size reduction of the *Lk6* mutant flies at 20% yeast is caused by a reduction in both cell size and cell number. Strikingly, *Lk6* mutant cell clones induced in larval imaginal discs grew poorly at 20% yeast as compared to their wild-type sibling clones, in sharp contrast to the normal growth at 100% yeast (compare Figure 3C with Figure 2C). We conclude that Lk6 is a positive growth regulator that becomes limiting only under poor nutrient supply.

We initially identified Lk6 as a negative growth regulator capable of suppressing, upon overexpression, a big-eye phenotype. Moreover, ubiquitous overexpression of Lk6 at 100% yeast resulted in a growth reduction (see above, Figures 1B and 1C). The results of the loss-of-function analysis suggest that Lk6 overexpression under our standard conditions acts in a dominant-negative manner. We wondered whether the negative effects on growth were also contingent on amino acid availability. At 30% and 20% yeast, Lk6-overexpressing flies were no longer reduced in weight when compared to control flies (Figure 3D). Furthermore, the lethality caused by *Act5CGal4*-mediated Lk6 overexpression at 100% yeast (only 16% and 2% survivors for females and males,

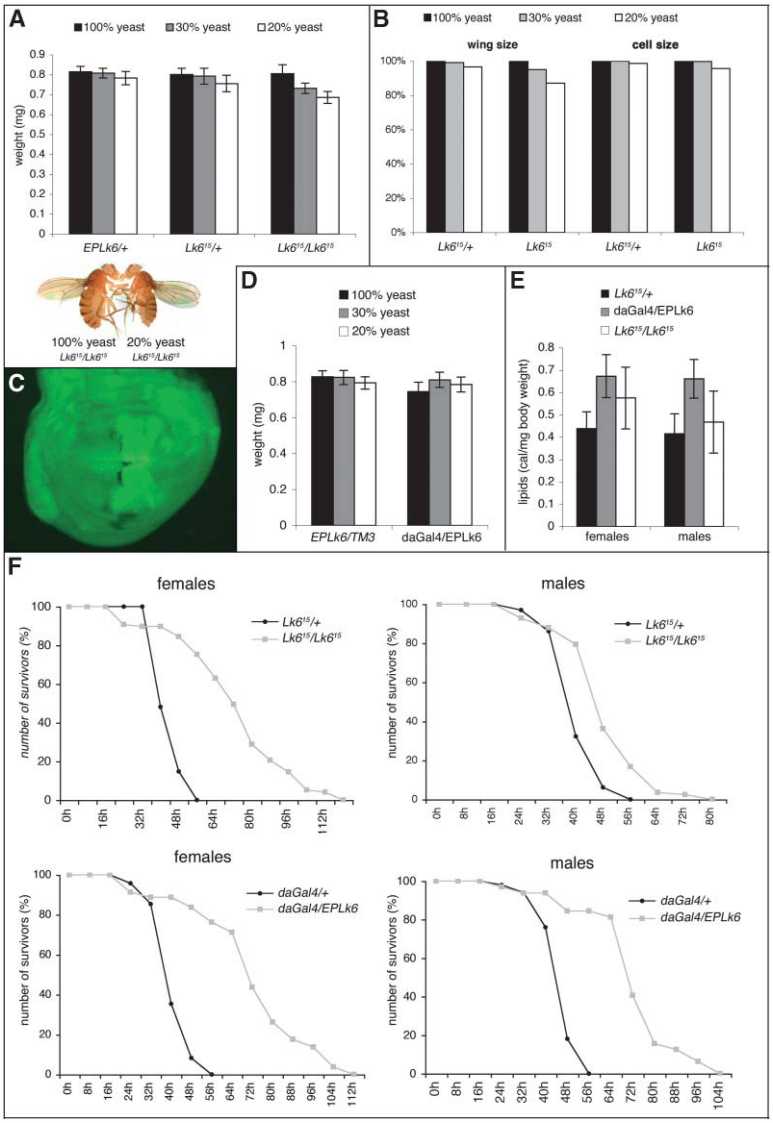

Figure 3. Reducing Dietary Yeast Reveals a Growth Function for Lk6

(A) Differential effects of reducing the yeast content in the food. Whereas control flies are of normal size when reared at 30% yeast, *Lk6* mutants are significantly smaller. Depending on the allelic combination, the weight reduction ranges from 9% to 17% in males and from 15% to 20% in females ($p < 0.001$; student' s t test). At 20% yeast, control flies are also slightly reduced in size (2%–6% in males, 3%–7% in females). Flies lacking Lk6 function are further reduced (15%–26% in males, 17%–29% in females). The weight of homozygous $Lk6^{15}$ males is shown as an example. Heterozygous $Lk6^{15}/+$ and *EPLk6*/+ males serve as controls. $n = 30–50$ for all measurements. A comparison of female *Lk6* mutant flies reared at 100% and 20% yeast, respectively, is shown below.

(B) Closer examination of adult wings reveals a predominant effect on cell number. Cell size is only affected at 20% yeast, both in the *Lk6* mutant and control flies. Ten wings of female flies were analyzed for each experimental condition, and mean values are displayed relative to the respective sizes at 100% yeast.

(C) At 20% yeast, clones of *Lk6* mutant cells (marked by the absence of GFP) bear a severe growth disadvantage in comparison to the wild-type sister clones (bright green). Genotype is the same as indicated in Figure 2C.

(D) The negative effects caused by ubiquitous overexpression of Lk6 on growth are diminished by reducing the yeast content in the food.

(E) Measurement of lipid levels in 3 day-old females and males (according to Bohni et al. [22]) reveals elevated lipid contents in Lk6-overexpressing flies (*y w;daGal4/EPLk6*; $n = 10$, $p < 0.05$) as well as in homozygous *Lk6* mutants ($Lk6^{15}$; $n = 9$, $p < 0.05$ for females).

(F) Both loss and gain of Lk6 function lead to an increase in the average lifespan of adult flies, as compared to control flies, under complete starvation ($n = 100$). Similar results were obtained with different *Lk6* alleles.

respectively) was substantially suppressed by rearing the animals on low-yeast food (74% and 16% survival ratio at 30% yeast, 58% and 8% at 20% yeast for females and males, respectively). From these overexpression experiments, we conclude that the activity of Lk6 is also regulated by amino acid availability.

Loss of Chico, the *Drosophila* homolog of vertebrate insulin receptor substrate 1-4 proteins (IRS1-4), results in flies with proportionally reduced body size [22]. These *chico* mutant flies have increased lipid levels and are more resistant to starvation than wild-type flies [23]. We therefore tested *Lk6* mutant animals for their behavior under starvation conditions and for changes in their lipid metabolism. The effect to sustain starvation (i.e., a water-only diet) was more pronounced in females: Adult $Lk6^{15}$ mutant females lived nearly 50% longer than control flies, $Lk6^{38}$ mutant females 33% longer (Figure 3F; data not shown). Male $Lk6^{15}$ and $Lk6^{38}$ mutants showed an increase in lifespan of 22% and 13%, respectively. Flies overexpressing Lk6 displayed a similar lifespan extension under fasting conditions (Figure 3F). Measurement of the lipid content in adult flies revealed significantly increased lipid levels in the mutant animals (+31% [loss-of-function] and +54% [overexpression] in females; +13% [loss-of-function] and +59% [overexpression] in males; Figure 3E), consistent with the notion that the enriched lipid stores can be mobilized to endure periods of starvation. Notably, lifespan of *Lk6* mutants was prolonged only under starvation conditions. Under normal culture conditions, *Lk6* mutant flies lived shorter than control flies (data not shown).

p38 MAPK is activated by diverse stress stimuli, including oxidative stress [6]. We therefore asked whether Lk6, as a p38 downstream effector, might be important for survival in response to oxidative stress. Adult *Lk6* mutants and control flies were subjected to an oxidative stress regime by feeding them a sucrose/paraquat mixture, which leads to the generation of reactive oxygen species when metabolized. *Lk6* mutants were more susceptible to paraquat feeding than control flies (Figure S2), indicating that Lk6 is critical for survival under oxidative stress conditions.

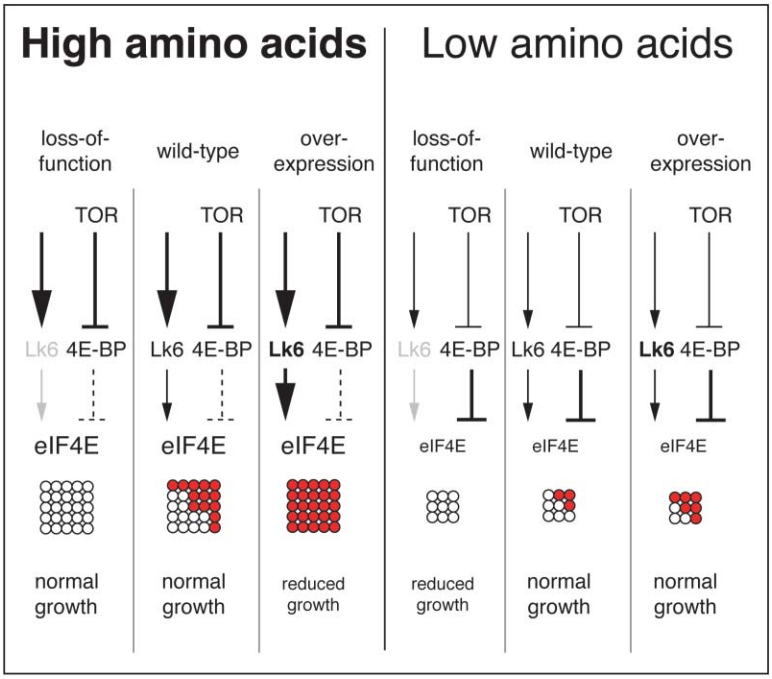

Figure 4. Effect of the Diet and Lk6 Dosage on Growth

TOR activity is stimulated under rich nutrient conditions (high amino acids), resulting in the phosphorylation of 4E-BP. This leads to an increase in the pool of free eIF4E (represented by dots), which assembles into functional translation initiation complexes. The translation efficiency is further modulated by phosphorylation of eIF4E by Lk6 (red dots symbolize phosphorylated eIF4E). A high amount of Lk6 results in complete phosphorylation of eIF4E and impairs growth because of premature eIF4E phosphorylation and/or a reduction of the affinity of phosphorylated eIF4E to capped mRNA. On the other hand, the loss of Lk6 is without impact on translation initiation under favorable conditions because phosphorylation of eIF4E is not essential as long as the pool of free eIF4E is sufficiently large. Under dietary restricted conditions, however, the absence of Lk6 leads to impaired translation efficiency. Lk6 activity is presumably also regulated in response to amino acid availability. See text for further details.

Conclusions

Our genetic analysis shows that Lk6 is dispensable under standard culture conditions (rich amino acid source), commensurate with the recent finding that Mnk1 and Mnk2 are not essential for cell growth and development in the mouse [4]. Under adverse food conditions, however, Lk6 is required for normal growth. It is interesting to note that Lk6 expression has been reported to be upregulated upon starvation during larval development [24].

We have also provided evidence that Lk6 exerts its function via phosphorylation of eIF4E because the effects of Lk6 overexpression are strictly dependent on the presence of Ser251 in eIF4E. This conclusion is strongly supported by the finding that eIF4E phosphorylation is diminished in ovaries of *Lk6* mutant flies [19]. Therefore, flies lacking Lk6 function can be expected to display the same phenotype as *eIF4E* mutant flies rescued by a *P{eIF4E^{Ser251Ala}}* transgene. However, the rescued *eIF4E* mutants grow to a smaller size even under our standard culture conditions. Although they contain significantly fewer cells, the size reduction is predominantly caused by smaller cells [12]. In contrast, the loss of Lk6 function primarily affects cell number. Whether these discrepancies reflect a qualitative difference between *eIF4E* mutant flies rescued by a *P{eIF4E^{Ser251Ala}}* transgene and *Lk6* mutants is currently unknown.

We speculate that the net result of Lk6/Mnk activity (i.e., whether translation is inhibited or promoted) is not determined by the absolute levels of Lk6/Mnk, but rather by the ratio of activated Lk6/Mnk and free eIF4E (i.e., not bound by 4E-BPs), the limiting factor for translation initiation (see Figure 4). Under our standard culture conditions (high protein), a larger fraction of eIF4E assembles into functional eIF4F complexes because of high TOR activity, thereby promoting translation. Under reduced conditions (e.g., 30% yeast), TOR pathway activity is lowered, and, thus, more 4E-BP binds and inhibits eIF4E, which dampens the rate of translation. It is likely that the predominant mechanism of eIF4E regulation is achieved by TOR/4E-BP activity, and that the phosphorylation of eIF4E by Lk6/Mnk imposes a translational fine-tuning that becomes rate limiting only under adverse food conditions. Lacking Lk6 function in addition to diminished eIF4E availability impinges on translation efficiency, which results in the observed body size reduction.

Alternatively, high TOR activity caused by a diet rich in amino acids could enable the activation of another (unidentified) eIF4E kinase that acts redundantly to Lk6/Mnk. However, this is rather unlikely because mice lacking Mnk1 and Mnk2 do not show any residual eIF4E-Ser209 phosphorylation, strongly arguing against an uncharacterized eIF4E kinase [4].

Overexpression of Lk6 under standard food conditions consistently resulted in a suppression of growth. Furthermore, another EP insertion in the *Lk6* locus (*EP3344*; see Experimental Procedures), which promotes lower expression levels as compared to *EPLk6*, yielded qualitatively similar but milder phenotypes (data not shown), suggesting that the dosage of Lk6 expression is important for its ability to regulate growth. Concentration-dependent effects of Mnk1 have also been described by Knauf et al. [15], who suggested a negative role of Mnk1/2 for cap-dependent translation. It is conceivable that overexpressed Lk6 exerts a dominant-negative effect on translation efficiency by reducing the affinity of phosphorylated eIF4E for capped mRNA, leading to a precocious disassembly of the eIF4F complex.

Reducing the amino acid supply abolished the negative effects of Lk6 overexpression on growth, suggesting that the activity of Lk6 is also regulated in response to nutrients. The mechanism for this additional layer of regulation is unknown but is likely to involve phosphorylation by the upstream kinases ERK and/or p38. Consis-

tently, the p38 homolog in fission yeast, Sty1/Spc1, is regulated in response to nutrient limitation and osmotic stress [25].

The effects of Lk6 activity are therefore context dependent: They lead either to growth stimulation or growth inhibition. We propose that (1) the timing, (2) the amount of eIF4E phosphorylation during 48S complex assembly, and (3) nutrient (amino acid) availability are critical parameters for the modulation of growth by Lk6.

Our results underscore the importance of the diet composition for growth studies. In fact, under the standard food conditions used in our laboratory, the *Lk6* mutant growth phenotype would have escaped detection, demonstrating that different food compositions are likely to result in qualitatively different outcomes of otherwise identical experiments. We feel that the researchers in the growth field, ourselves included, have not paid sufficient attention to what their flies actually eat. Therefore, we would like to propose that *Drosophila* geneticists should always describe the composition of the fly food when reporting on growth-related experiments. Ideally, a global standard fly medium should be defined.

The relationship between nutrition and growth has also been appreciated by others [26]. It has been estimated that 14%–20% of all cancer deaths in the US are attributable to overweight and obesity [27]. Therefore, understanding the role of diet in cancer development will represent a crucial task in the future.

Experimental Procedures

Insertion Sites of EP Elements

The insertion site of *EP30.18* (=*EPLk6*) was determined by plasmid rescue after EcoRI digestion of the genomic DNA. *EP30.18* is inserted ~1950 bp upstream of the *Lk6* open reading frame (ORF) encoding the shorter isoform. Another predicted *Lk6* 5′ splice variant gives rise to a putative protein with an addition of 52 amino acids (without any recognizable domains) at the N terminus. *EP(3)3344* was obtained from the Szeged stock center. It is inserted ~3180 bp further upstream of *EPLk6* and ~1030 bp upstream of the start codon of the longer *Lk6* splice variant, and it enables lower Lk6 expression than *EPLk6*, as assessed by in situ hybridization (data not shown).

Fly Food Composition

Our standard fly food contains the following ingredients per 1 liter water: 75 g glucose/dextrose, 55 g maize, 10 g flour, 8 g agar, 15 ml nipagin (33 g/l EtOH)/nipasol (66 g/l EtOH), and 100 g live yeast. The dietary-restricted food (30% or 20% yeast) contains 30 g and 20 g yeast per 1 liter water, respectively.

Starvation Assay

Freshly eclosed flies were separated according to sex and kept on standard fly food for 3 days before they were transferred to empty plastic vials sealed with water-soaked foam stoppers. The vials were kept at 25°C, and dead flies were counted every 4 or 8 hr.

Supplemental Data

Supplemental figures are available at http://www.current-biology.com/cgi/content/full/15/1/24/DC1/.

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References

1. Lazaris-Karatzas, A., Montine, K.S., and Sonenberg, N. (1990). Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. Nature *345*, 544–547.
2. De Benedetti, A., and Harris, A.L. (1999). eIF4E expression in tumors: Its possible role in progression of malignancies. Int. J. Biochem. Cell Biol. *31*, 59–72.
3. Scheper, G.C., Morrice, N.A., Kleijn, M., and Proud, C.G. (2001). The mitogen-activated protein kinase signal-integrating kinase Mnk2 is a eukaryotic initiation factor 4E kinase with high levels of basal activity in mammalian cells. Mol. Cell. Biol. *21*, 743–754.
4. Ueda, T., Watanabe-Fukunaga, R., Fukuyama, H., Nagata, S., and Fukunaga, R. (2004). Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development. Mol. Cell. Biol. *24*, 6539–6549.
5. Waskiewicz, A.J., Flynn, A., Proud, C.G., and Cooper, J.A. (1997). Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. EMBO J. *16*, 1909–1920.
6. Roux, P.P., and Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: A family of protein kinases with diverse biological functions. Microbiol. Mol. Biol. Rev. *68*, 320–344.
7. Rorth, P. (1996). A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. Proc. Natl. Acad. Sci. USA *93*, 12418–12422.
8. Rorth, P., Szabo, K., Bailey, A., Laverty, T., Rehm, J., Rubin, G.M., Weigmann, K., Milan, M., Benes, V., Ansorge, W., et al. (1998). Systematic gain-of-function genetics in Drosophila. Development *125*, 1049–1057.
9. Kidd, D., and Raff, J.W. (1997). LK6, a short lived protein kinase in Drosophila that can associate with microtubules and centrosomes. J. Cell Sci. *110*, 209–219.
10. Huang, A.M., and Rubin, G.M. (2000). A misexpression screen identifies genes that can modulate RAS1 pathway signaling in Drosophila melanogaster. Genetics *156*, 1219–1230.
11. Gingras, A.C., Raught, B., and Sonenberg, N. (1999). eIF4 initiation factors: Effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. *68*, 913–963.
12. Lachance, P.E., Miron, M., Raught, B., Sonenberg, N., and Lasko, P. (2002). Phosphorylation of eukaryotic translation initiation factor 4E is critical for growth. Mol. Cell. Biol. *22*, 1656–1663.
13. Scheper, G.C., and Proud, C.G. (2002). Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? Eur. J. Biochem. *269*, 5350–5359.
14. McKendrick, L., Morley, S.J., Pain, V.M., Jagus, R., and Joshi, B. (2001). Phosphorylation of eukaryotic initiation factor 4E (eIF4E) at Ser209 is not required for protein synthesis in vitro and in vivo. Eur. J. Biochem. *268*, 5375–5385.
15. Knauf, U., Tschopp, C., and Gram, H. (2001). Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2. Mol. Cell. Biol. *21*, 5500–5511.
16. Minich, W.B., Balasta, M.L., Goss, D.J., and Rhoads, R.E. (1994). Chromatographic resolution of in vivo phosphorylated and nonphosphorylated eukaryotic translation initiation factor eIF-4E: Increased cap affinity of the phosphorylated form. Proc. Natl. Acad. Sci. USA *91*, 7668–7672.
17. Scheper, G.C., van Kollenburg, B., Hu, J., Luo, Y., Goss, D.J., and Proud, C.G. (2002). Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. J. Biol. Chem. *277*, 3303–3309.
18. Zuberek, J., Wyslouch-Cieszynska, A., Niedzwiecka, A., Dadlez, M., Stepinski, J., Augustyniak, W., Gingras, A.C., Zhang, Z., Burley, S.K., Sonenberg, N., et al. (2003). Phosphorylation of

eIF4E attenuates its interaction with mRNA 5′ cap analogs by electrostatic repulsion: Intein-mediated protein ligation strategy to obtain phosphorylated protein. RNA *9*, 52–61.

19. Arquier, N., Bourouis, M., Colombani, J., and Léopold, P. (2005). *Drosophila* Lk6 kinase controls phosphorylation of Eukaryotic Translation Initiation Factor 4E and promotes normal growth and development. Curr. Biol. *15*, this issue, 19–23.
20. Kim, D.H., and Sabatini, D.M. (2004). Raptor and mTOR: Subunits of a nutrient-sensitive complex. Curr. Top. Microbiol. Immunol. *279*, 259–270.
21. Gingras, A.C., Raught, B., and Sonenberg, N. (2001). Regulation of translation initiation by FRAP/mTOR. Genes Dev. *15*, 807–826.
22. Bohni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., and Hafen, E. (1999). Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1–4. Cell *97*, 865–875.
23. Oldham, S., Stocker, H., Laffargue, M., Wittwer, F., Wymann, M., and Hafen, E. (2002). The Drosophila insulin/IGF receptor controls growth and size by modulating PtdInsP(3) levels. Development *129*, 4103–4109.
24. Zinke, I., Schutz, C.S., Katzenberger, J.D., Bauer, M., and Pankratz, M.J. (2002). Nutrient control of gene expression in Drosophila: Microarray analysis of starvation and sugar-dependent response. EMBO J. *21*, 6162–6173.
25. Shiozaki, K., and Russell, P. (1995). Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. Nature *378*, 739–743.
26. Manson, M.M. (2003). Cancer prevention—the potential for diet to modulate molecular signalling. Trends Mol. Med. *9*, 11–18.
27. Calle, E.E., Rodriguez, C., Walker-Thurmond, K., and Thun, M.J. (2003). Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N. Engl. J. Med. *348*, 1625–1638.

Discussion

The control of growth and size – an unsolved mystery

How Are the Sizes of Cells, Organs, and Bodies Controlled?

Ernst Hafen and Hugo Stocker

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Unsolved Mystery

How Are the Sizes of Cells, Organs, and Bodies Controlled?

Ernst Hafen* and Hugo Stocker

Why is an elephant bigger than a mouse? Why, luckily, are our arms precisely the same size? While developmental genetics over the past 20 years has provided us with fascinating insights into how segments form, limbs bud, and axons find their targets, we have made little progress towards answering these obvious questions in biology. Rather than attempting to provide the answers, we will try to frame the questions in a developmental context and highlight some approaches towards answering them. Somewhat artificially, we will consider separately the mechanisms of cell, organ, and body size control.

What Controls the Size of Eukaryotic Cells?

The size of a cell depends on intrinsic and extrinsic factors. For example, cell size can vary dramatically with cell type—some neurons or glia cells are up to 1,000 times larger than epithelial cells. Cell size is also influenced by the number of genome sets (ploidy). A haploid *Drosophila* epithelial cell is only about half the size of a diploid cell. A polyploid salivary gland cell, on the other hand, is more than 1,000 times larger than a diploid cell. Amongst the extrinsic factors, the availability of nutrients and temperature are well known for their effect on cell size. Starvation not only prolongs the cell doubling time in yeast and in *Drosophila* cells, it also reduces the size at which they divide.

Work from Zetterberg (Killander and Zetterberg 1965) in mammalian fibroblasts and subsequently from Nurse and Hartwell in yeast provided evidence for a cell size checkpoint (Nurse 1975; Johnston et al. 1977). In budding yeast, the protein Cln3p acts as a sizer. Cells only initiate the critical cell cycle step from G1 phase to S phase, when Cln3p has reached a certain threshold. The accumulation of Cln3p is, in turn, dependent on efficient translation of the *Cln3* mRNA, which is inefficiently translated until sufficient numbers of ribosomes have been generated (Polymenis and Schmidt 1997). In this way, the presence of an efficient translation machinery is a prerequisite for passing the cell size checkpoint. Indeed, in a whole-genome survey of mutants affecting cell size in budding yeast, many size mutants exhibited defects in ribosome biogenesis (Jorgensen et al. 2002).

Ribosome biogenesis also appears to be an important regulator of cell size in multicellular organisms. Phosphorylation of the ribosomal protein S6 by S6 kinase (S6K) results in the preferential translation of ribosomal proteins and thus in the replenishment of the protein synthesis machinery. *Drosophila* cells lacking functional S6K grow more slowly and are smaller than normal cells, possibly because of the earlier accumulation of a cell sizer analogous to Cln3p in yeast (Thomas 2000).

Unsolved Mysteries discuss a topic of biological importance that is poorly understood and in need of attention.

Abbreviations: *brk*, *brinker*; Dpp, Decapentaplegic; Hh, Hedgehog; IGF, insulin-like growth factor; *M*, *Minute*; S6K, S6 kinase; Shh, Sonic hedgehog; TGFβ, transforming growth factor β; Tkv, Thickveins

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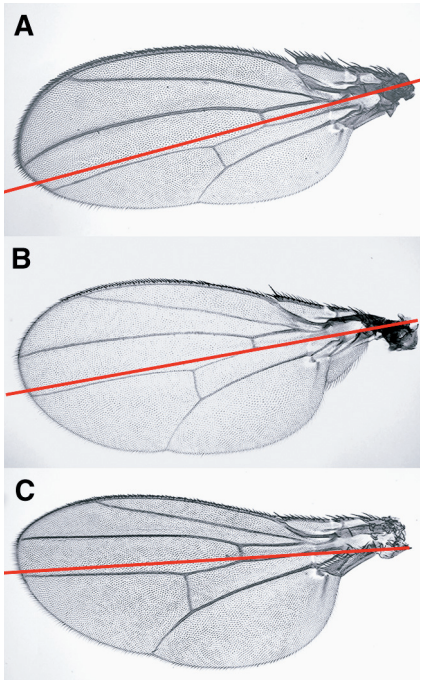

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Figure 1. The Insulin Signaling Activity Controls Organ Size in a Compartment-Specific Manner

Mosaic *Drosophila* wings with compartment-specific manipulations of dAkt function display striking size defects but normal patterning.

(A) Selective reduction of dAkt function in the posterior compartment by means of FLP-mediated mitotic recombination in posterior cells (using *engrailed–Gal4* to drive the expression of *UAS–Flp*) results in a small P compartment largely consisting of $dAkt^{3}$ mutant cells. The smaller compartment size is due to fewer and smaller cells.

(B) Wild-type wing for comparison.

(C) Expression of dAkt in posterior cells (*engrailed–Gal4 UAS–dAkt*) of wings with reduced dAkt function ($dAkt^{3}$) restores the size of the P compartment, whereas the A compartment remains small. The red lines mark the anterior–posterior compartment boundary. Note that similar results in the wing disc have been obtained by Teleman and Cohen (2000).

But is there a need for a cell size checkpoint in multicellular organisms? One would assume so, because otherwise cells would either become progressively smaller or larger. However, it has been suggested that this may be a problem for exponentially growing cells like yeast, but that mammalian cell growth is linear and, under these conditions, the need for a cell size checkpoint may be less stringent. Indeed, Conlon and Raff (2003) did not observe a cell size checkpoint in rat Schwann cells grown under different growth factor conditions (see also Grewal and Edgar 2003). Furthermore, the existence of cell size checkpoints may be cell-type dependent and stage specific. During *Drosophila* imaginal disc development, for example, cells are larger at the beginning of imaginal disc growth and become progressively smaller during later stages (Madhavan and Schneiderman 1977).

Until recently, more emphasis has been placed on understanding the genetic control of cell cycle progression than on the mechanisms regulating cell growth. This has often led to the use of cell proliferation and cell growth as synonymous terms. Analysis in *Drosophila* imaginal discs using cell clones either deficient in cell cycle progression or expressing cell cycle regulators that accelerate or slow down the cell cycle, however, have shown clearly that cell cycle progression alone is not sufficient to promote growth (Weigmann et al. 1997; Neufeld et al. 1998). In summary, cell size is altered by changing ploidy, by uncoupling cell cycle progression from cell growth, and by pathways regulating cell growth such as the insulin (see below) and S6K pathways. Of these three, only the modulation of cell growth has an effect on overall growth at the next level, the organ.

How Is the Size of Organs Controlled?

Changes in organ size are only partly due to changes in cell size. In *Drosophila*, the reduction in wing size in S6K mutant flies or in flies raised at higher temperature is caused by a reduction in cell size (Partridge et al. 1994; Montagne et al. 1999); in contrast, starvation or mutations in genes coding for insulin signaling components that mediate the starvation response affect body size and organ size by reducing cell size and cell number (Garofalo 2002). The effect of insulin pathway activity on growth is largely autonomous to cells and multicellular regions, called compartments. Specific reduction of dAkt function, an essential component of the insulin signaling pathway, in either the anterior or the posterior compartment of the wing imaginal disc results in a severe reduction of the respective compartment. Astonishingly, the small compartment is properly patterned and the size and patterning of the adjacent compartment remain untouched (Figure 1), demonstrating that the insulin pathway has a profound effect on the final size of an organ without interfering with the patterning mechanism.

Recently, a novel signaling complex that restricts organ size by controlling both proliferation arrest and apoptosis has been discovered (Ryoo and Steller 2003). Mutations in either *hippo*, *salvador*, or *warts* result in a failure of cell cycle exit and in a protection from cell death, thus leading to massively overgrown organs. How an organ *knows* when it has reached its final size, however, is still mysterious and thus challenging.

It is clear that autonomous and nonautonomous factors control organ size, but their relative contribution varies depending on organ type and species. Multiple transplanted fetal thymus glands each grow to their normal size while multiple transplanted fetal spleens grow collectively to the size of one spleen (reviewed in Conlon and Raff 1999). In *Drosophila*, immature imaginal discs (larval structures that undergo metamorphosis and develop into structures such as legs, wings, and eyes in the adult) transplanted into a third instar larva do not undergo metamorphosis until they reach the final size (Bryant and Simpson 1984). But the size of insect appendages is not only controlled autonomously. Ablation of the hind wing discs in butterflies increases the size of the fore wings (Nijhout and Emlen 1998).

The Role of Cell Competition

Based on experiments in mammalian systems, it has been suggested that the competition for limiting growth or survival factors may be a general mechanism for organ size control (Conlon and Raff 1999). In *Drosophila*, cell competition is observed in imaginal discs. Slowly growing cells are eliminated when they are next to cells that grow at a normal rate (Simpson and Morata 1981). The slowly growing cells in these studies were heterozygous at one of several *Minute* (*M*) loci, some of which encode ribosomal proteins.

Recently, a link has been established

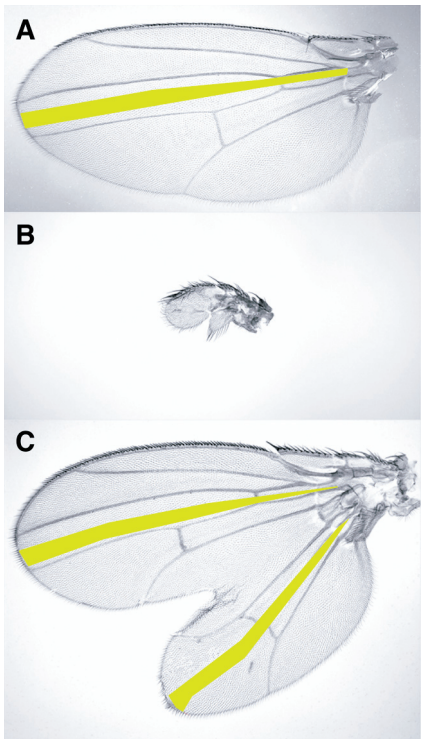

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Figure 2. Changing the Patterning Mechansims during Wing Development Affects Growth
Compared with a wild-type wing (A), loss of Dpp function results in reduced growth and loss of pattern elements (B). Ectopic expression of Dpp in a clone of cells results in pattern duplications associated with massive extra growth (C). The region of Dpp expression in (A) and (C) is indicated by the green color. (Zecca et al. 1995; pictures courtesy of B. Müller and K. Basler.)

between cell competition and signaling by the secreted factor Decapentaplegic (Dpp) (Moreno et al. 2002). The elimination of slowly growing *M/+* cells is preceded by the upregulation of the gene *brinker* (*brk*), which triggers cell death. Expression of *brk* is downregulated by high Dpp levels. As in *M/+* cells, *brk* upregulation and cell elimination by apoptosis are also triggered in cells close to the Dpp source that are unresponsive to Dpp because they lack the Dpp receptor Thickveins (Tkv). Slowly growing cells may be outcompeted because they may be less efficient in internalizing Dpp via endocytosis and thus receive fewer survival signals. The problem with this simple model is that cells away from the anterior–posterior boundary—the site of Dpp production—possess high levels of Brk but do not die and grow at the same rate as cells close to the Dpp source. Indeed, *tkv* mutant clones also survive in these regions (Burke and Basler 1996). Therefore, *brk* levels do not correlate with the growth and survival potential of cells in all circumstances.

An alternative explanation for the observed parallels between the elimination of *tkv* mutant cells and *M/+* cells is that the juxtaposition of cells with different cell surface properties is the trigger for elimination. The upregulation of *brk* in *M/+* cells may trigger different surface properties (positional identities) in the same way as in *tkv* mutant cells. Thus, cell competition may be a cell-policing mechanism that eliminates cells that for various reasons do not fit into the community. Whether this mechanism of cell competition plays a major role in organ size control is still unclear.

How Are Pattern Formation and Growth Connected?

Organ size is coupled to pattern formation. Interfering with patterning mechanisms, for example, by implanting a bead soaked in the secreted factor Sonic hedgehog (Shh) into the anterior of the chick wing bud or by the ectopic expression of Hedgehog (Hh) or Dpp in the *Drosophila* wing, causes pattern duplications and concomitant growth. Conversely, partial loss-of-function mutations in *dpp* reduce wing size (Potter and Xu 2001) (Figure 2). In contrast to the effects caused by modulating insulin pathway activity, the stimulation of growth by Dpp appears to be tightly linked with pattern formation. How is patterning coupled to growth? This is one of the major unsolved questions in the field. It does not appear that the patterning morphogens like Dpp act by directly promoting growth since the cell division rates are the same in regions of high and low Dpp concentrations (Milan et al. 1996).

An attractive hypothesis put forward based on a previous model of regeneration postulates that the individual cells of an organ primordium measure the concentration gradients of specific signaling molecules, such as Dpp in the *Drosophila* wing disc and Shh in the vertebrate limb bud (Day and Lawrence 2000). In immature small primordia, the gradients are steep and cells continue to grow and divide. Since the source of the gradient stays approximately constant, its concentration gradient flattens as the tissue grows. When the difference in the morphogen concentration sensed by the two ends of the cells along the axis of the gradient falls below a certain threshold, the cells stop growing. Although this model could explain why cell growth and division are not concentrated around

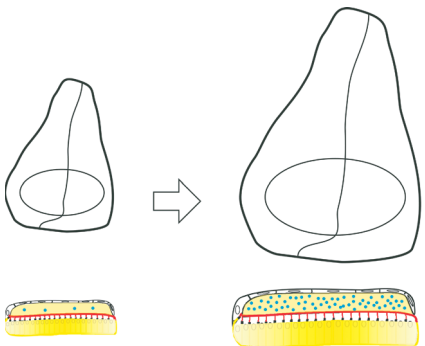

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Figure 3. Model for the Coordinated Control of Growth and Patterning in the *Drosophila* Wing Disc
A schematic representation of a growing (left) and a mature (right) wing disc is shown at the top. Corresponding cross-sections through the wing blade region are depicted below. The wing disc originates from the infolding of the embryonic ectoderm and consists of pseudostratified epithelial cells containing a basal–lateral side (yellow) and an apical side (red). The apical surface faces the disc lumen that is formed by the epithelium and the overlaying peripodial membrane (black), consisting of squamous epithelial cells. The morphogen and growth factor Dpp (yellow) is secreted basal–laterally by the Dpp-producing cells located anterior to the anterior–posterior compartment boundary (line through centre of wing disc). The Dpp concentration gradient from the anterior–posterior boundary to the periphery provides the anterior–posterior patterning cues. In addition, Dpp is also secreted apically into the disc lumen where is can diffuse freely. The model proposes that luminal Dpp acts as a growth-promoting factor stimulating disc growth in young discs. As the disc grows, a hypothetical growth inhibitor (blue dots) is also secreted apically and antagonizes the growth promoting activity of Dpp. Once the concentration of the inhibitor has reached a certain threshold, proliferation of wing imaginal disc cells ceases.

sources of morphogens, experimental evidence does not support it. Clones of cells expressing a constitutively active version of the Dpp receptor Tkv show increased growth when surrounded by cells of low Tkv activity. Furthermore, constant overexpression of activated Tkv in the entire disc also promotes growth, arguing against growth being induced by a differential of Tkv activity across the cell (Lecuit et al. 1996; Nellen et al. 1996).

How then does normal graded Tkv activity produce homogenous growth? One possible solution to this problem comes from the observation that Dpp in the *Drosophila* wing is secreted basal–laterally as well as apically (Figure 3). While Dpp secreted on the basal–lateral side in the epithelium has been detected in a concentration gradient (Teleman and Cohen 2000), Dpp secreted on the apical side accumulates in the disc lumen formed by the disc epithelium proper and the peripodial membrane, whose cells also secrete Dpp (Gibson et al. 2002). It is tempting to speculate that Dpp in the lumen functions as a general growth-promoting factor, while Dpp secreted in a graded fashion from the basal–lateral side induces pattern formation. A growth-promoting function has been suggested for the luminally produced Dpp (Gibson et al. 2002). This model implies that Dpp received on the apical side of the cell triggers a different cellular response (growth, survival, or both) than Dpp received on the basal–lateral side (patterning) and would probably require an unequal distribution of Dpp receptors or signaling components along the apical–basal axis of the cell.

At present, the most attractive hypothesis for how intrinsic control of organ size is achieved postulates that a secreted growth-promoting factor accumulates in the organ primordium and that its function is counteracted by an inhibitor accumulating with a delay (Nijhout 2003). Once the inhibitor reaches a certain threshold and/or the growth factor is consumed, organ growth stops (Figure 3). Although hypothetical, activator and inhibitor models have been postulated for many patterning processes. Dpp and related transforming growth factor β (TGFβ) molecules provide a particularly well-established case. TGFβ agonists and antagonists are involved in patterning the dorsal–ventral axis in the *Drosophila* embryo and the left–right asymmetry in the vertebrate embryos (Capdevila and Belmonte 1999). Further genetic and biochemical experiments are needed to identify the components involved in intrinsic organ growth control.

Which Growth Promoting Pathways Are Regulated by Secreted Factors with Patterning Functions?

Although little is known about the connection between patterning factors and growth pathways, a few potential links have been described. For example, in the *Drosophila* eye imaginal disc, Hh regulates growth directly by controlling the expression of cyclin E, a promoter of the G1/S transition, and by cyclin D, a promoter of cell growth (Duman-Scheel et al. 2002). Whether this is a general mechanism by which Hh controls cell growth and cell division is unclear, however, since in the wing disc at least, the effect of Hh appears to be mediated by Dpp. Comprehensive surveys of target genes regulated by these patterning factors in the specific developing tissues using microarray technology may provide further insight into how they control cell growth directly or indirectly.

How Is Body Size Controlled?

Can the question of body size regulation be reduced to simply summing up the mechanisms that regulate the size of individual organs? In contrast to organ size control that involves local cell interactions, locally produced growth factors as well as systemic growth factors, overall body size is controlled primarily by systemic factors. Vertebrate body size is controlled by growth hormone and the subordinate insulin-like growth factors (IGFs) (Butler and Roith 2001). In invertebrates, growth and body size are also regulated by the insulin/IGF system in response to nutrients. In addition, final body size in insects is determined by the number of molting cycles, and these are under the control of the steroid hormone ecdysone and the sesquiterpenoid juvenile hormone (Nijhout 2003). Nevertheless, changing ecdysone or insulin-like peptide levels in invertebrates or overproducing growth hormone in vertebrates can increase body size only within a certain range. It is obviously not possible to turn a mouse into the size of an elephant, although the recent identification of fossils of *Phoberomys pattersoni* indicates that rodents were once a great deal larger than they are today (Sanchez-Villagra et al. 2003). In addition to the hormonal control of body size, there are intrinsic genetic constraints to organ and body size. Understanding the mechanism underlying these constraints will be another challenge for the future.

Conclusions

In contrast to the control of cell fate, segment number, or patterning, which is largely determined by genetic regulatory mechanisms, the control of size is influenced by genetic, hormonal, and environmental inputs. Understanding this phenomenon requires a combination of developmental genetic, physiological, and evolutionary approaches. Given the significant interest that has been generated in growth control, it should not be long before some of these old mysteries in biology are explained. This will not only reward us with a better understanding of this important aspect of developmental biology, but it will also provide better insight into human diseases, such as cancer, that are associated with a misregulation of cellular growth. ■

References

Bryant PJ, Simpson P (1984) Intrinsic and extrinsic control of growth in developing organs. Q Rev Biol 59: 387–415.

Burke R, Basler K (1996) Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing. Development 122: 2261–2269.

Butler AA, Roith DL (2001) Control of growth by the somatropic axis: Growth hormone and the insulin-like growth factors have related and independent roles. Annu Rev Physiol 63: 141–164.

Capdevila J, Belmonte JC (1999) Extracellular modulation of the Hedgehog, Wnt and TGF-beta signalling pathways during embryonic development. Curr Opin Genet Dev 9: 427–433.

Conlon I, Raff M (1999) Size control in animal development. Cell 96: 235–244.

Conlon I, Raff M (2003) Differences in the way a mammalian cell and yeast cells coordinate cell growth and cell cycle progression. J Biol 2: 7.

Day SJ, Lawrence PA (2000) Measuring dimensions: The regulation of size and shape. Development 127: 2977–2987.

Duman-Scheel M, Weng L, Xin S, Du W (2002) Hedgehog regulates cell growth and proliferation by inducing cyclin D and cyclin E. Nature 417: 299–304.

Garofalo RS (2002) Genetic analysis of insulin signaling in *Drosophila*. Trends Endocrinol Metab 13: 156–162.

Gibson MC, Lehman DA, Schubiger G (2002) Lumenal transmission of *decapentaplegic* in Drosophila imaginal discs. Dev Cell 3: 451–460.
Grewal S, Edgar BA (2003) Controlling cell division in yeast and animals: Does size matter? J Biol 2: 5.
Johnston GC, Pringle JR, Hartwell LH (1977) Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. Exp Cell Res 105: 79–98.
Jorgensen P, Nishikawa JL, Breitkreutz BJ, Tyers M (2002) Systematic identification of pathways that couple cell growth and division in yeast. Science 297: 395–400.
Killander D, Zetterberg A (1965) A quantitative cytochemical investigation of the relationship between cell mass and initiation of DNA synthesis in mouse fibroblasts *in vitro*. Exp Cell Res 40: 12–20.
Lecuit T, Brook WJ, Ng M, Calleja M, Sun H, et al. (1996) Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing. Nature 381: 387–393.
Madhavan MM, Schneiderman HA (1977) Histological analysis of the dynamics of growth of imaginal discs and histoblast nests during the larval development of *Drosophila melanogaster*. J Roux Arch Dev Biol 183: 269–305.
Milan M, Campuzano S, Garcia-Bellido A (1996) Cell cycling and patterned cell proliferation in the *Drosophila* wing during metamorphosis. Proc Natl Acad Sci U S A 93: 11687–11692.
Montagne J, Stewart M, Stocker H, Hafen E, Kozma S, et al. (1999) *Drosophila* S6 kinase: A regulator of cell size. Science 285: 2126–2129.
Moreno E, Basler K, Morata G (2002) Cells compete for *decapentaplegic* survival factor to prevent apoptosis in Drosophila wing development. Nature 416: 755–759.
Nellen D, Burke R, Struhl G, Basler K (1996) Direct and long-range action of a *dpp* morphogen gradient. Cell 85: 357–368.
Neufeld TP, Delacruz AFA, Johnston LA, Edgar BA (1998) Coordination of growth and cell division in the Drosophila wing. Cell 93: 1183–1193.
Nijhout HF (2003) The control of body size in insects. Dev Biol 261: 1–9.
Nijhout HF, Emlen DJ (1998) Competition among body parts in the development and evolution of insect morphology. Proc Natl Acad Sci U S A 95: 3685–3689.
Nurse P (1975) Genetic control of cell size at cell division in yeast. Nature 256: 547–551.
Partridge L, Barrie B, Fowler K, French V (1994) Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. Evolution 48: 1269–1276.
Polymenis M, Schmidt EV (1997) Coupling of cell division to cell growth by translational control of the G1 cyclin *CLN3* in yeast. Genes Dev 11: 2522–2531.
Potter CJ, Xu T (2001) Mechanisms of size control. Curr Opin Genet Dev 11: 279–286.
Ryoo HD, Steller H (2003) Hippo and its mission for growth control. Nat Cell Biol 5: 853–855.
Sanchez-Villagra MR, Aguilera O, Horovitz I (2003) The anatomy of the world's largest extinct rodent. Science 301: 1678–1679.
Simpson P, Morata G (1981) Differential mitotic rates and patterns of growth in compartments in the *Drosophila* wing. Dev Biol 85: 299–308.
Teleman AA, Cohen SM (2000) Dpp gradient formation in the Drosophila wing imaginal disc. Cell 103: 971–980.
Thomas G (2000) An encore for ribosome biogenesis in the control of cell proliferation. Nat Cell Biol 2: E71–E72.
Weigmann K, Cohen SM, Lehner CF (1997) Cell cycle progression, growth and patterning in imaginal discs despite inhibition of cell division after inactivation of *Drosophila* Cdc2 kinase. Development 124: 3555–3563.
Zecca M, Basler K, Struhl G (1995) Sequential organizing activities of *engrailed*, *hedgehog*, and *decapentaplegic* in the *Drosophila* wing. Development 121: 2265–2278.

Drosophila melanogaster – a model system to study growth

How is cellular growth regulated? What mechanisms ensure that growth is in tune with environmental conditions? At which size does a cell have to divide? How do organs know when they have reached their appropriate size? Who conducts the orchestra of cell birth and cell death during the development of multicellular organisms? While some of these issues have been addressed in cell culture experiments, there is doubtlessly an immediate need for genetically amenable model organisms to gain insight into the mysteries of growth control. *Drosophila melanogaster* appears to be well suited for this purpose. The development of the imaginal discs provides an ideal setup for the analysis of growth processes. Imaginal discs are single-layered epidermal tissues that grow during larval development to eventually give rise to the adult appendages. Importantly, imaginal disc cells remain diploid (in contrast to most larval cells), and their cell cycles include G1 and G2 phases (Edgar and Lehner, 1996), much as mammalian cells do. Furthermore, the cell division patterns during imaginal disc development appear to be rather random, sharply contrasting the situation in another simple genetic model organism, *Caenorhabditis elegans*. In this nematode, development follows a relatively strict cell lineage. As a result, the number of cells in an adult animal is astonishingly constant, suggesting that *C. elegans* mainly governs growth by controlling the cell divisions. Therefore, the control of growth in imaginal discs of *Drosophila* resembles the mammalian situation much closer. What are the advantages of *Drosophila* over the mouse system? One striking difference is the relative ease with which genetic analyses can be performed in the fly. Not only is the generation time of *Drosophila* considerably shorter, but fly geneticists also have many more sophisticated tools in hands. Moreover, redundancy appears to be less of a problem in *Drosophila* as compared to mammals. For instance, while three structurally and functionally related insulin receptor-like receptor tyrosine kinases exist in the mouse (IR, IGF-IR, and IRR), there is only one fly ortholog (Inr). The four mammalian IRS molecules also have a unique *Drosophila* counterpart. In the case of Akt, the single fly kinase has three orthologs in the mouse. The discrepancy in genetic complexity is further increased by the apparently much greater variety of protein isoforms derived from mammalian genes. Therefore, genetic investigations in the low-complexity system (*Drosophila*) are much more promising, whereas analyses in the mouse are inherently ambiguous. On the other hand, it can be argued that the simple setup of

Drosophila might not adequately reflect the multilayered control mechanisms governing mammalian development. The hope of the fly geneticists is, therefore, that the signaling pathways emerging from their studies constitute the heart of an evolutionarily conserved system, which is partially covered by additional layers of control in higher organisms such as mammals.

Akt/PKB is the major effector of PI3K signaling during *Drosophila* development

One goal of the present work was to clarify the role of the proto-oncogene Akt during *Drosophila* development. In light of the wealth of studies on Akt function in mammalian cell culture systems, this analysis might appear to be superfluous. However, most cell culture experiments suffer from severe disadvantages. One obvious drawback is the isolation of cells from their natural context. Such isolation might for instance have a tremendous impact on the ability to survive under delicate conditions. Moreover, cell culture studies are often based on overexpression of particular protein variants. The inherent danger of overexpression experiments is that the expression level might exceed physiological concentrations, thereby possibly causing unwanted side effects. In the case of so-called dominant negative protein variants, the mechanism of action often remains obscure, further complicating the interpretation of the observed effects. Thus, an *in vivo* analysis of the gene function by genetic means is imperative.

Akt function has been investigated in the nematode *C. elegans*. It could be shown that the two Akt genes (*akt-1* and *akt-2*) act in the insulin receptor pathway downstream of the PI3K ortholog AGE-1 (Paradis and Ruvkun, 1998). Since a mutation in the forkhead transcription factor DAF-16 relieves the requirement for Akt activity, the *akt* genes primarily function to antagonize DAF-16 (Paradis and Ruvkun, 1998), indicating that the signaling pathway downstream of the insulin receptor DAF-2 might be rather linear in *C. elegans*. While these studies in *C. elegans* confirm the participation of Akt in the insulin receptor signaling cascade, they do not tell us anything about Akt's role in growth control.

Akt function has also been analyzed in knockout mice. Mice lacking Akt1 (PKBα) displayed defects in embryonic and postnatal growth (Chen et al., 2001; Cho et al., 2001b), whereas a loss of Akt2 (PKBβ) function resulted in metabolic defects

consistent with a role of Akt2 in insulin action (Cho et al., 2001a). Therefore, the functions of the different Akt isoforms appear to be complementary rather than redundant. Surprisingly, the effects of Akt knockouts on cell survival were mild. Only in some tissues (testis, thymus) of mice devoid of Akt1, increased spontaneous apoptosis could be observed (Chen et al., 2001). *Akt1* $^{-/-}$ mouse embryonic fibroblasts (MEFs) were more susceptible to cell death triggered by various inducers (Chen et al., 2001). The unexpectedly weak apoptosis phenotype could be explained in two ways. Either Akt's anti-apoptotic function is redundant (shared by at least two of the three isoforms), or the results of various cell culture experiments were overstated. Double knockout mice for *Akt1* and *Akt2* have been reported only recently (Peng et al., 2003). They are severely growth-impaired and die shortly after birth, displaying similar phenotypes as *IGF-IR* knock out mice (defects in skin and bone development and severe skeletal muscle atrophy), suggesting that Akt1/2 are the major downstream effectors of IGF-1R.

Redundancy should not perturb genetic analyses in *Drosophila*, as the fly genome contains a single gene encoding an Akt homolog. However, two isoforms with apparent molecular weights of 66 and 85 kDa, respectively, are generated by the alternative usage of an upstream non-AUG translational initiation signal (Andjelkovic et al., 1995). It is presently unclear whether the less abundant longer isoform (with an addition of 81 amino acids at its NH2-terminus) exerts functions distinct from those of the more common shorter isoform.

Flies lacking Akt function were shown to die during larval stages, and removal of the maternal contribution led to excessive cell death during embryogenesis (Staveley et al., 1998). A closer look at Akt's function during larval development revealed that Akt is not essential for cellular survival, but for cellular growth. Clones consisting of cells devoid of functional Akt remain tiny, but, importantly, they are not out-competed by surrounding faster dividing cells. Contrary to the expectation, these cells are somehow protected from apoptosis and they are even able to properly differentiate. Flies with reduced Akt activity develop slowly and emerge as small adults. They consist of fewer cells of reduced size. This phenotype is reminiscent of the effects of starvation (Simpson, 1979).

In another series of experiments, it could be demonstrated that the activity of the insulin receptor signal not only dictates growth and size at the level of single cells, but also at the levels of compartments and organs. For instance, reducing Akt function specifically in the posterior (P) compartment of the developing wing gives

rise to an adult wing with a smaller P compartment, while the anterior compartment remains unaffected (Hafen and Stocker, 2003). Although much reduced in size, the P compartment exhibits a surprisingly normal wing vein pattern. It is difficult to explain how the same source of the morphogen Dpp is able to elicit fundamentally different responses in the two compartments. Further experimentation will be needed to address this issue. Removal of Akt function from the entire eye-antennal imaginal discs (by means of the ey-flp technique (Newsome et al., 2000)) results in flies with tiny eyes and heads on bodies of normal size. Thus, whole organs grow to a size that is in accordance with the signal strength of the insulin receptor pathway within the organ, irrespective of the other organs.

The characterization of a hypomorphic *Akt* allele (in collaboration with Mirjana Andjelkovic, FMI, Basel) revealed a reduced affinity of the mutant kinase for PIP3 due to a single amino acid exchange in the PH domain. This mutation was instrumental in testing the importance of Akt as a mediator of the growth-stimulating effects caused by the loss of the tumor suppressor PTEN. In the absence of PTEN function, the concentration of the second messenger PIP3 raises. Accumulation of PIP3 is believed to recruit a variety of proteins containing PIP3-interacting domains (such as the PH domain) to the plasma membrane. In many cases, the relocalization of the protein is expected to result in a stimulation of its activity. Therefore, it was generally assumed that the simultaneous activation of numerous effectors would sum up to the complete response elicited by the loss of PTEN function. A simple genetic experiment proved the contrary, at least in *Drosophila*. Flies devoid of PTEN function and with measurably increased PIP3 concentrations can readily survive if only the affinity of Akt for PIP3 is reduced. The generalization of this finding will certainly be questioned. Possibly, signaling downstream of the second messenger PIP3 is far less complicated in the fly than it is in mammals. For instance, a number of PH domain-containing proteins (e.g. DAPP1, TAPP1, and TAPP2) are not encoded by the *Drosophila* genome. Alternatively, it could equally well be that the importance of other potential effectors has been overestimated. The severe phenotype of *Akt1 Akt2* double knockout mice is suggesting that Akt also plays a major role in IGF-IR mediated growth in mammals.

The genetic identification of novel insulin signaling components

The highly characteristic pinhead phenotype of flies lacking Akt function specifically in the progenitor cells of the head and the eye laid the foundations for a mutagenesis screen aiming at the identification of novel components of insulin receptor signaling as well as of other genes involved in growth control. Using the elegant ey-flp technique developed by Barry Dickson (Newsome et al., 2000), mutations in growth-regulating genes could be easily identified in the first generation by virtue of the resultant abnormal head size phenotype. The right arm of the third chromosome was screened to saturation, and numerous flies displaying a pinhead phenotype were isolated. Interestingly, flies exhibiting the opposite phenotype did also emerge. Larger than normal heads can form if the function of a negative regulator of a growth-promoting signal is impaired, or if a proliferation-blocking signal is missing.

The focus of the screen was on the potential novel components of the insulin receptor pathway. We found six complementation groups that fulfill the crucial criteria for positive insulin signaling components (the mutant tissue grows poorly but can properly differentiate). Three of the candidate groups correspond to the backbone of the signal transduction cascade: The receptor (Inr), the lipid kinase PI3K that produces the second messenger PIP3, and the major effector of PIP3, Akt/PKB. Alleles of the three other groups carry mutations in the genes encoding the small GTPase Rheb (see below), MASK and Lnk, respectively. MASK (multiple ankyrin repeats, single KH domain) is a huge protein with an RNA binding domain (KH domain). *MASK* mutations have also been isolated as dominant enhancers of a phenotype caused by the expression of dominant-negative Corkscrew (Smith et al., 2002). Corkscrew encodes a protein tyrosine phosphatase that is required for signaling downstream of receptor tyrosine kinases such as Sevenless and the epidermal growth factor receptor (EGFR). Consistently, clones of *MASK* mutant cells displayed phenotypes reminiscent of reduced EGFR signaling (impaired proliferation, differentiation and survival). Interestingly, the mutations identified in our screen do not severely impinge on photoreceptor differentiation. Either our mutations represent hypomorphic alleles, or they specifically compromise a particular (growth) function of MASK while leaving other functions (differentiation) intact. The identification of the molecular lesions might help in resolving this issue.

All heteroallelic combinations of *Lnk* alleles are viable and yield flies of reduced size, reminiscent of (although milder than) the *chico* mutant phenotype (Bohni et al., 1999). The mammalian Lnk family of adaptor proteins consists of Lnk, APS (adaptor protein with a PH domain and an SH2 domain) and SH2-B. Besides the characteristic PH and SH2 domains, all family members (including *Drosophila* Lnk) contain potential tyrosine phosphorylation sites that serve as docking sites for the adaptor molecule Grb2 as well as proline-rich stretches. By virtue of binding to phosphatidylinositols, the PH domain directs the adaptors to membranes where the SH2 domain mediates binding to phospho-tyrosines, e.g. motifs in autophosphorylated receptor tyrosine kinases. Not only is the modular composition of Lnk family members strikingly similar to that of the IRS proteins, but APS and SH2-B have also been shown to function as an insulin receptor substrates (Ahmed et al., 1999; Kotani et al., 1998; Moodie et al., 1999). They could, therefore, be functionally redundant with IRS proteins. APS binds the activated IR much stronger than SH2-B, and expression of APS or SH2-B correlates with sustained signaling activity (Ahmed and Pillay, 2003). It has been speculated that APS and SH2-B might stabilize the active conformation of the IR, or they might prevent its dephosphorylation (Ahmed and Pillay, 2003). The generation of knockout mice revealed functions of APS and SH2-B in insulin signaling. Mice devoid of functional SH2-B were slightly growth-retarded and failed to build proper genital organs (Ohtsuka et al., 2002). Furthermore, they developed insulin resistance and glucose intolerance during aging (Duan et al., 2004), suggesting that SH2-B is a physiological enhancer of insulin receptor activity. Surprisingly, the disruption of the *APS* gene resulted in increased insulin sensitivity (Minami et al., 2003), which could be explained by the increased serum levels of leptin and adiponectin in these mice. These strikingly different knockout phenotypes demonstrate that APS and SH2-B cannot functionally substitute each other in vivo. In *Drosophila*, the IRS homolog Chico is the only adaptor protein binding to Inr described so far. Interestingly, *chico* is not an essential gene, in contrast to the genes encoding Inr, Dp110/PI3K and Akt/PKB. This could be explained in two ways. Either basal PI3K activation occurs via the COOH-terminal extension of the *Drosophila* Inr, or the signal is mediated by another adaptor protein. Lnk is an obvious candidate for the latter case. Interestingly, flies lacking *chico* are no longer able to survive if they carry one mutant copy of *Lnk*, indicative of a strong genetic interaction between *chico* and *Lnk*. It is also tempting to speculate that Lnk might bridge between the Inr and Ras-MAPK signaling via interaction with Drk/Grb2.

Although the *Inr* loss-of-function phenotype is not indicative of a reduction in Ras-MAPK signal strength, the overexpression of Inr results in phosphorylation of MAPK (Brogiolo et al., 2001). It will be interesting to test whether this crosstalk depends on functional Lnk.

Negative regulators of insulin signaling are expected to result in a bighead phenotype in the ey-flp assay. An example is the tumor suppressor PTEN: Loss of PTEN in mosaic animals gives rise to enlarged cells and tissues (Gao et al., 2000; Goberdhan et al., 1999; Huang et al., 1999). All the novel mutations that displayed a very similar phenotype fell into a single complementation group corresponding to the *Tsc1* locus. The TSC1 (Tuberous sclerosis complex 1, also called hamartin) and TSC2 (Tuberous sclerosis complex 2, also known as tuberin) tumor suppressor proteins are both required to form a functional complex, and mutations in both genes are linked to a hamartomas syndrome called tuberous sclerosis (hence the names). Whereas TSC2 bears a GAP (GTPase activating protein) domain, TSC1 hooks the complex to the membrane via its transmembrane domain. Studies on Tsc1/2 function in *Drosophila* implied this tumor suppressor as a component of the insulin receptor signal transduction cascade or of a pathway acting in parallel (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). Furthermore, a negative role of Tsc1/2 in the regulation of TOR (target of Rapamycin) activity was reported (Gao et al., 2002; Inoki et al., 2002), and a direct link between Inr signaling and the Tsc1/2 complex via phosphorylation of Tsc2 by Akt was suggested (Inoki et al., 2002; Potter et al., 2002). However, a recent study suggests that this phosphorylation is not a physiological event, but rather occurs under high insulin signaling activity only (Dong and Pan, 2004).

Is Tsc2 the only target of Akt/PKB? Data from mammalian cell culture suggest that members of the FOXO family of transcription factors are excluded from the nucleus upon phosphorylation by Akt (Kops et al., 1999). In fact, the only *Drosophila* FOXO homolog gets phosphorylated by Akt upon insulin treatment, leading to an increased expression of 4EBP and Inr (Junger et al., 2003; Puig et al., 2003). The expression of Inr constitutes a novel feedback regulation of unknown function (Puig et al., 2003). A genetic analysis of *FOXO* mutants did not reveal any growth defects in flies with otherwise normal insulin signaling. It was only when insulin signaling activity was lowered (e.g. by *chico* or hypomorphic *Akt* mutations) that the loss of FOXO exerted

positive effects on growth, resulting in a partial suppression of the growth deficit (Junger et al., 2003). Interestingly, the suppressive effect was solely due to an effect on cell number. Moreover, flies lacking Akt function could almost survive in the absence of functional FOXO, indicating that FOXO is indeed a critical target of Akt (Junger et al., 2003). How can these findings be reconciled with the notion that Akt signals primarily via Tsc2 (Potter et al., 2002)? It is conceivable that the primary target of Akt depends on signaling strength. Whereas abnormally high signaling activity results in hyperactivation of Akt and subsequent inactivation of the Tsc1/Tsc2 complex, physiological levels of Akt activity are required to keep FOXO in check.

The small GTPase Rheb is negatively regulated by the tumor suppressors Tsc1/Tsc2 and controls TOR activity

Mutations in the gene encoding the homolog of the small GTPase Rheb (Ras homolog enriched in brain) were isolated based on the resulting pinhead phenotype. Hypomorphic *Rheb* mutants (in certain heteroallelic combinations) were reduced in size, and the females were sterile. *Rheb* mutant cells grew poorly but they were capable of properly differentiating. Conversely, overexpression of Rheb promoted cellular growth. All these characteristics also apply to insulin signaling components such as Inr, Dp110/PI3K and Akt/PKB. Therefore, it was reasonable to assume that Rheb is a component of the insulin signaling cascade. A detailed epistasis analysis placed Rheb downstream of Tsc1/2 and upstream of TOR and S6K. The fact that flies lacking Tsc1 function can be rescued by reducing Rheb activity suggests that the inactivation of Rheb is the main task of Tsc1/2. These genetic findings were corroborated by the demonstration that Tsc2 is the Rheb GAP (Garami et al., 2003; Inoki et al., 2003a; Tee et al., 2003; Zhang et al., 2003), and that S6K activity critically depends on Rheb. Our observation that *Rheb* is also epistatic over *PTEN* is consistent with Akt talking to Tsc2 under conditions of elevated PIP3 levels.

The regulation of Rheb mainly occurs via its GAP, Tsc2. No Rheb GEF has been reported so far. How is the activity of Tsc2 controlled? Phosphorylation at various regulatory sites appears to be key. Besides Akt/PKB, other kinases such as AMPK (Inoki et al., 2003b) and MK2 (Li et al., 2003) can also phosphorylate Tsc2. Since AMPK is activated by low cellular energy levels (and thereby increased AMP

concentration), AMPK provides a link between energy status of the cell and TOR activity. Interestingly, full AMPK activation depends on phosphorylation by LKB1, a tumor suppressor mutated in patients with Peutz-Jeghers syndrome, establishing a link between the tumor suppressors LKB1 and TSC1/2 (Corradetti et al., 2004; Shaw et al., 2004a; Shaw et al., 2004b). Therefore, TSC2 appears to be an integrator of diverse signals, coupling cellular conditions to the activation of TOR.

Rheb function correlates with the activity of S6K, a well-established (direct?) downstream target of TOR. This raises the question how Rheb regulates TOR, a huge multidomain (NH2-terminal HEAT domains) serine/threonine kinases that is inhibited by the drug rapamycin (Schmelzle and Hall, 2000). Genetic analyses in the fly demonstrated that the loss of TOR gives rise to similar growth effects as seen for *Rheb* mutants (Neufeld, 2004; Oldham et al., 2000; Zhang et al., 2000), consistent with the idea that the activation of TOR is the main outcome of Rheb action. In *Saccharomyces cerevisiae*, TOR exists in high molecular weight complexes of two flavors (Loewith et al., 2002). TOR Complex 1 (TORC1), which is Rapamycin sensitive, contains TOR1 or TOR2, KOG1 and LST8, whereas TOR Complex 2 (TORC2), which is not inhibited by rapamycin, is composed of TOR2, AVO1-3 and LST8. TORC2 is not implicated in growth regulation; it rather acts on the actin cytoskeleton. In mammals, two similar complexes appear to exist (although higher eukaryotes have only one TOR). Raptor (regulatory associated protein of mTOR) that shares homology with KOG1 is essential for TOR's growth function (Hara et al., 2002; Kim and Sabatini, 2004; Kim et al., 2002). The mammalian homolog of LST8, mLST8 or GßL, is also part of the TOR complex (Kim et al., 2003). It strongly binds to the kinase domain of mTOR and stabilizes the interaction of Raptor with mTOR. Interestingly, the sensitivity to nutrients and rapamycin depends on the presence of GßL in the complex (Kim et al., 2003). Recently, a mammalian counterpart of AVO3 was shown to be part of a TOR complex. Rictor (rapamycin-insensitive companion of mTOR) forms a complex with mTOR that neither contains Raptor, nor is it sensitive to rapamycin (Sarbassov dos et al., 2004). The Rictor-mTOR complex also signals to the actin cytoskeleton, raising the possibility that the TORC2 is conserved from yeast to man. In this context, it is noteworthy that clones of *Rheb* mutant cells display unusual elongated shapes, a behavior that could be explained by the tendency of *Rheb* mutant cells to minimize contact to each other. It is tempting to speculate that the altered adhesion behavior is mediated by TORC2. Future experiments will be

directed towards the identification of Rheb's effectors that contribute to the regulation of the two TOR complexes.

How conserved are the insulin and TOR signaling pathways?

Signaling triggered by the activation of the insulin receptor appears to be a highly conserved signal transduction pathway that regulates growth in accordance with environmental conditions. In the nematode *C. elegans*, the cascade headed by the insulin-receptor DAF-2 controls development and longevity. Under adverse environmental conditions (food shortage, high temperature or high population density), the worms arrest development in the dauer stage. This diapause state allows long-term survival, and the subsequent adult lifespan is not affected by the period spent in the dauer stage. Mutations in the positively acting components of the insulin receptor pathway result in a dauer-constitutive phenotype, whereas worms lacking DAF-18/PTEN never enter the dauer stage. Hypomorphic mutations in *daf-2* or *age-1* significantly extend the adult lifespan (Kenyon et al., 1993), and the focus of insulin receptor signaling in the control of lifespan was shown to reside in the CNS (Apfeld and Kenyon, 1998; Wolkow et al., 2000). The ability of insulin receptor signaling to coordinate development with environmental conditions also applies to *Drosophila*. The phenotype of flies with reduced Inr signaling activity is indistinguishable from that of flies reared under adverse conditions (small body size, delay in development). Recently, a direct influence of amino acid deprivation on the formation of the second messenger PIP3 could be demonstrated (Britton et al., 2002). Does the functional conservation of insulin receptor signaling extend further? The situation in mammals appears to be more complex. Clearly, insulin plays a crucial role in the regulation of glucose homeostasis and metabolism, whereas the IGFs are primarily involved in growth regulation. It is conceivable that the mammalian ligands have adopted more specialized functions during evolution. Nevertheless, there are striking parallels between insulin secretion in the pancreatic ß-cells and the production of several DILPs in neurosecretory cells of the *Drosophila* brain (Ikeya et al., 2002; Rulifson et al., 2002), and this analogy has recently been further extended to the glucose sensing mechanism and the production of the insulin antagonist glucagon (Kim and Rulifson, 2004). Therefore, the picture in lower metazoans might highlight the ancient functions of the insulin signal transduction cascade. This raises

the question whether a similar mechanism can already be found in lower organisms such as yeast. The fact that no orthologs of the insulin receptor or of PI3K are encoded by the yeast genomes suggests that no similar signaling pathway involving the second messenger PIP3 exists. However, recent findings by Fabrizio and colleagues are very intriguing (Fabrizio et al., 2001). They identified mutations in SCH9, the yeast ortholog of Akt, based on their resistance to heat and oxidative stress in mutant nondividing cells. This could indicate that the downstream components of the insulin receptor signaling cascade are more highly conserved as compared to the top of the hierarchy. A similar constellation has been proposed for the sex determining pathways across species (Wilkins, 1995). In fact, the TOR signaling branch is highly conserved from yeast to man (see above). Rheb also exists in yeast, and it has been implicated in the regulation of amino acid uptake (Urano et al., 2000). In *Schizosaccharomyces pombe*, the TSC1/2 complex negatively regulates Rheb to control arginine uptake (Van Slegtenhorst et al., 2004). It remains to be determined whether the activity of the TSC1/2 complex in yeast is regulated by kinases with homology to Akt, AMPK and MK2.

The high evolutionary conservation of insulin signaling in higher eukaryotes also has medical implications. Deregulation of insulin signaling causes severe diseases such as diabetes and cancer. In fact, the tumor suppressor PTEN is frequently lost in a variety of cancer types (Cantley and Neel, 1999; Simpson and Parsons, 2001). Therefore, the identification of novel signaling components in model organisms such as *C. elegans* and *Drosophila melanogaster* might enhance the understanding of human diseases and eventually lead to novel therapeutic interventions. From the data presented in this thesis, it can be deduced that many tumors could potentially be attacked in two ways: Either by molecules specifically disturbing the interaction of Akt's PH domain with the second messenger PIP3, or by the inhibition of Rheb activity (e.g. by farnesyltransferase inhibitors).

Drosophila has told us a great deal about growth control. There are, however, numerous unsolved issues to be addressed in the future. How does the fly sense nutrients? Do the DILPs respond to energy levels? Which aspects of insulin receptor signaling are (non)autonomous? Do we know all the target genes of Akt's kinase activity? How are the insulin and TOR signaling pathways connected? What is the mechanism of TOR activation by Rheb? How do amino acids regulate the TOR complex? These are some of the questions that immediately arise from the findings of our recent research. In the long term, more complex issues like the enigmatic relationship of patterning and growth control will attract our attention. *Drosophila* promises to be the leading edge in this exciting field of research.

References

Accili, D., Drago, J., Lee, E. J., Johnson, M. D., Cool, M. H., Salvatore, P., Asico, L. D., Jose, P. A., Taylor, S. I., and Westphal, H. (1996). Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. Nat Genet *12*, 106-109.

Ahmed, Z., and Pillay, T. S. (2003). Adapter protein with a pleckstrin homology (PH) and an Src homology 2 (SH2) domain (APS) and SH2-B enhance insulin-receptor autophosphorylation, extracellular-signal-regulated kinase and phosphoinositide 3-kinase-dependent signalling. Biochem J *371*, 405-412.

Ahmed, Z., Smith, B. J., Kotani, K., Wilden, P., and Pillay, T. S. (1999). APS, an adapter protein with a PH and SH2 domain, is a substrate for the insulin receptor kinase. Biochem J *341*, 665-668.

Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997). Role of translocation in the activation and function of protein kinase B. J Biol Chem *272*, 31515-31524.

Andjelkovic, M., Jones, P. F., Grossniklaus, U., Cron, P., Schier, A. F., Dick, M., Bilbe, G., and Hemmings, B. A. (1995). Developmental regulation of expression and activity of multiple forms of the Drosophila RAC protein kinase. J Biol Chem *270*, 4066-4075.

Apfeld, J., and Kenyon, C. (1998). Cell nonautonomy of C. elegans daf-2 function in the regulation of diapause and life span. Cell *95*, 199-210.

Araki, E., Lipes, M. A., Patti, M. E., Bruning, J. C., Haag, B., 3rd, Johnson, R. S., and Kahn, C. R. (1994). Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature *372*, 186-190.

Baker, J., Liu, J. P., Robertson, E. J., and Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. Cell *75*, 73-82.

Bathgate, R. A., Samuel, C. S., Burazin, T. C., Gundlach, A. L., and Tregear, G. W. (2003). Relaxin: new peptides, receptors and novel actions. Trends Endocrinol Metab *14*, 207-213.

Bathgate, R. A., Samuel, C. S., Burazin, T. C., Layfield, S., Claasz, A. A., Reytomas, I. G., Dawson, N. F., Zhao, C., Bond, C., Summers, R. J.*, et al.* (2002). Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. Novel members of the relaxin peptide family. J Biol Chem *277*, 1148-1157.

Birnbaum, M. J. (2001). Turning down insulin signaling. J Clin Invest *108*, 655-659.

Bogatcheva, N. V., Truong, A., Feng, S., Engel, W., Adham, I. M., and Agoulnik, A. I. (2003). GREAT/LGR8 is the only receptor for insulin-like 3 peptide. Mol Endocrinol *17*, 2639-2646.

Bohni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B. F., Beckingham, K., and Hafen, E. (1999). Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. Cell *97*, 865-875.

Britton, J. S., Lockwood, W. K., Li, L., Cohen, S. M., and Edgar, B. A. (2002). Drosophila's Insulin/PI3-Kinase Pathway Coordinates Cellular Metabolism with Nutritional Conditions. Dev Cell *2*, 239-249.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., and Hafen, E. (2001). An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. Curr Biol *11*, 213-221.

Burger, A. M., Zhang, X., Li, H., Ostrowski, J. L., Beatty, B., Venanzoni, M., Papas, T., and Seth, A. (1998). Down-regulation of T1A12/mac25, a novel insulin-like growth factor binding protein related gene, is associated with disease progression in breast carcinomas. Oncogene *16*, 2459-2467.

Cantley, L. C., and Neel, B. G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci U S A *96*, 4240-4245.

Carpenter, C. L., Auger, K. R., Duckworth, B. C., Hou, W. M., Schaffhausen, B., and Cantley, L. C. (1993). A tightly associated serine/threonine protein kinase regulates phosphoinositide 3-kinase activity. Mol Cell Biol *13*, 1657-1665.

Chen, C., Jack, J., and Garofalo, R. S. (1996). The Drosophila insulin receptor is required for normal growth. Endocrinology *137*, 846-856.

Chen, W. S., Xu, P. Z., Gottlob, K., Chen, M. L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K.*, et al.* (2001). Growth retardation and increased apoptosis in mice with homozygous disruption of the akt1 gene. Genes Dev *15*, 2203-2208.

Chiang, S. H., Baumann, C. A., Kanzaki, M., Thurmond, D. C., Watson, R. T., Neudauer, C. L., Macara, I. G., Pessin, J. E., and Saltiel, A. R. (2001). Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. Nature *410*, 944-948.

Cho, H., Mu, J., Kim, J. K., Thorvaldsen, J. L., Chu, Q., Crenshaw, E. B., 3rd, Kaestner, K. H., Bartolomei, M. S., Shulman, G. I., and Birnbaum, M. J. (2001a). Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science *292*, 1728-1731.

Cho, H., Thorvaldsen, J. L., Chu, Q., Feng, F., and Birnbaum, M. J. (2001b). Akt1/PKBa is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J Biol Chem *31*, 31.

Conlon, I., and Raff, M. (1999). Size control in animal development. Cell *96*, 235-244.

Corradetti, M. N., Inoki, K., Bardeesy, N., DePinho, R. A., and Guan, K. L. (2004). Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. Genes Dev *18*, 1533-1538.

Datta, S. R., Brunet, A., and Greenberg, M. E. (1999). Cellular survival: a play in three Akts. Genes Dev *13*, 2905-2927.

DeChiara, T. M., Efstratiadis, A., and Robertson, E. J. (1990). A growth-deficiency phenotype in heterozygous mice carrying an insulin- like growth factor II gene disrupted by targeting. Nature *345*, 78-80.

Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M. J., Gout, I., Totty, N. F., Truong, O., Vicendo, P., Yonezawa, K., and et al. (1994). PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity. Embo J *13*, 522-533.

Dong, J., and Pan, D. (2004). Tsc2 is not a critical target of Akt during normal Drosophila development. Genes & Development *18*, in press.

Dschietzig, T., and Stangl, K. (2003). Relaxin: a pregnancy hormone as central player of body fluid and circulation homeostasis. Cell Mol Life Sci *60*, 688-700.

Duan, C., Yang, H., White, M. F., and Rui, L. (2004). Disruption of the SH2-B gene causes age-dependent insulin resistance and glucose intolerance. Mol Cell Biol *24*, 7435-7443.

Edgar, B. A., and Lehner, C. F. (1996). Developmental control of cell cycle regulators: a fly's perspective. Science *274*, 1646-1652.

Efstratiadis, A. (1998). Genetics of mouse growth. Int J Dev Biol *42*, 955-976.

Fabrizio, P., Pozza, F., Pletcher, S. D., Gendron, C. M., and Longo, V. D. (2001). Regulation of longevity and stress resistance by Sch9 in yeast. Science *292*, 288-290.

Fantin, V. R., Wang, Q., Lienhard, G. E., and Keller, S. R. (2000). Mice lacking insulin receptor substrate 4 exhibit mild defects in growth, reproduction, and glucose homeostasis. Am J Physiol Endocrinol Metab *278*, E127-133.

Ferlin, A., Simonato, M., Bartoloni, L., Rizzo, G., Bettella, A., Dottorini, T., Dallapiccola, B., and Foresta, C. (2003). The INSL3-LGR8/GREAT ligand-receptor pair in human cryptorchidism. J Clin Endocrinol Metab *88*, 4273-4279.

Fernandez, R., Tabarini, D., Azpiazu, N., Frasch, M., and Schlessinger, J. (1995). The Drosophila insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential. EMBO J *14*, 3373-3384.

Finch, C. E., and Ruvkun, G. (2001). The genetics of aging. Annu Rev Genomics Hum Genet *2*, 435-462.

Gao, X., Neufeld, T. P., and Pan, D. (2000). Drosophila PTEN regulates cell growth and proliferation through PI3K- dependent and -independent pathways. Dev Biol *221*, 404-418.

Gao, X., and Pan, D. (2001). TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. Genes Dev *15*, 1383-1392.

Gao, X., Zhang, Y., Arrazola, P., Hino, O., Kobayashi, T., Yeung, R. S., Ru, B., and Pan, D. (2002). Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. Nat Cell Biol *4*, 699-704.

Garami, A., Zwartkruis, F. J., Nobukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S. C., Hafen, E., Bos, J. L., and Thomas, G. (2003). Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell *11*, 1457-1466.

Goberdhan, D. C. I., Paricio, N., Goodman, E. C., Mlodzik, M., and Wilson, C. (1999). Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. Genes Dev *13*, 3244-3258.

Gu, J., Tamura, M., Pankov, R., Danen, E. H., Takino, T., Matsumoto, K., and Yamada, K. M. (1999). Shc and FAK differentially regulate cell motility and directionality modulated by PTEN. J Cell Biol *146*, 389-403.

Guarente, L., and Kenyon, C. (2000). Genetic pathways that regulate ageing in model organisms. Nature *408*, 255-262.

Guarente, L., Ruvkun, G., and Amasino, R. (1998). Aging, life span, and senescence. Proc Natl Acad Sci U S A *95*, 11034-11036.

Hafen, E., and Stocker, H. (2003). How Are the Sizes of Cells, Organs, and Bodies Controlled? PLoS Biol *1*, E86.

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J., and Yonezawa, K. (2002). Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell *110*, 177-189.

Hsu, S. Y., Nakabayashi, K., Nishi, S., Kumagai, J., Kudo, M., Sherwood, O. D., and Hsueh, A. J. (2002). Activation of orphan receptors by the hormone relaxin. Science *295*, 671-674.

Huang, H., Potter, C. J., Tao, W., Li, D. M., Brogiolo, W., Hafen, E., Sun, H., and Xu, T. (1999). PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development. Development *126*, 5365-5372.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., and Hafen, E. (2002). Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. Curr Biol *12*, 1293-1300.

Inoki, K., Li, Y., Xu, T., and Guan, K. L. (2003a). Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev *17*, 1829-1834.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K. L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol *4*, 648-657.

Inoki, K., Zhu, T., and Guan, K. L. (2003b). TSC2 mediates cellular energy response to control cell growth and survival. Cell *115*, 577-590.

Joshi, R. L., Lamothe, B., Cordonnier, N., Mesbah, K., Monthioux, E., Jami, J., and Bucchini, D. (1996). Targeted disruption of the insulin receptor gene in the mouse results in neonatal lethality. Embo J *15*, 1542-1547.

Junger, M. A., Rintelen, F., Stocker, H., Wasserman, J. D., Vegh, M., Radimerski, T., Greenberg, M. E., and Hafen, E. (2003). The Drosophila Forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J Biol *2*, 20.

Kato, M. V. (2000). A secreted tumor-suppressor, mac25, with activin-binding activity. Mol Med *6*, 126-135.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993). A C. elegans mutant that lives twice as long as wild type. Nature *366*, 461-464.

Kido, Y., Burks, D. J., Withers, D., Bruning, J. C., Kahn, C. R., White, M. F., and Accili, D. (2000). Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. J Clin Invest *105*, 199-205.

Kim, D. H., and Sabatini, D. M. (2004). Raptor and mTOR: subunits of a nutrient-sensitive complex. Curr Top Microbiol Immunol *279*, 259-270.

Kim, D. H., Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell *110*, 163-175.

Kim, D. H., Sarbassov dos, D., Ali, S. M., Latek, R. R., Guntur, K. V., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2003). GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol Cell *11*, 895-904.

Kim, S. K., and Rulifson, E. J. (2004). Conserved mechanisms of glucose sensing and regulation by Drosophila corpora cardiaca cells. Nature *431*, 316-320.

Kitamura, T., Kahn, C. R., and Accili, D. (2003). Insulin receptor knockout mice. Annu Rev Physiol *65*, 313-332.

Kitamura, T., Kido, Y., Nef, S., Merenmies, J., Parada, L. F., and Accili, D. (2001). Preserved pancreatic beta-cell development and function in mice lacking the insulin receptor-related receptor. Mol Cell Biol *21*, 5624-5630.

Komatsu, S., Okazaki, Y., Tateno, M., Kawai, J., Konno, H., Kusakabe, M., Yoshiki, A., Muramatsu, M., Held, W. A., and Hayashizaki, Y. (2000). Methylation and downregulated expression of mac25/insulin-like growth factor binding protein-7 is associated with liver tumorigenesis in SV40T/t antigen transgenic mice, screened by restriction landmark genomic scanning for methylation (RLGS-M). Biochem Biophys Res Commun *267*, 109-117.

Kops, G. J., de Ruiter, N. D., De Vries-Smits, A. M., Powell, D. R., Bos, J. L., and Burgering, B. M. (1999). Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature *398*, 630-634.

Kotani, K., Wilden, P., and Pillay, T. S. (1998). SH2-Balpha is an insulin-receptor adapter protein and substrate that interacts with the activation loop of the insulin-receptor kinase. Biochem J *335 (Pt 1)*, 103-109.

Krajnc-Franken, M. A., van Disseldorp, A. J., Koenders, J. E., Mosselman, S., van Duin, M., and Gossen, J. A. (2004). Impaired nipple development and parturition in LGR7 knockout mice. Mol Cell Biol *24*, 687-696.

Kulkarni, R. N., and Okada, T. (2002). Tissue-specific targeting of the insulin receptor gene. Endocrine *19*, 257-266.

Kumagai, J., Hsu, S. Y., Matsumi, H., Roh, J. S., Fu, P., Wade, J. D., Bathgate, R. A., and Hsueh, A. J. (2002). INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent. J Biol Chem *277*, 31283-31286.

Lawlor, M. A., and Alessi, D. R. (2001). PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? J Cell Sci *114*, 2903-2910.

Lee, Y. H., and White, M. F. (2004). Insulin receptor substrate proteins and diabetes. Arch Pharm Res *27*, 361-370.

Leevers, S. J., Vanhaesebroeck, B., and Waterfield, M. D. (1999). Signalling through phosphoinositide 3-kinases: the lipids take centre stage. Current Opinion in Cell Biology *11*, 219-225.

Leevers, S. J., Weinkove, D., MacDougall, L. K., Hafen, E., and Waterfield, M. D. (1996). The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. EMBO J *15*, 6584-6594.

Li, Y., Inoki, K., Vacratsis, P., and Guan, K. L. (2003). The p38 and MK2 kinase cascade phosphorylates tuberin, the tuberous sclerosis 2 gene product, and enhances its interaction with 14-3-3. J Biol Chem *278*, 13663-13671.

Liu, C., Chen, J., Sutton, S., Roland, B., Kuei, C., Farmer, N., Sillard, R., and Lovenberg, T. W. (2003a). Identification of relaxin-3/INSL7 as a ligand for GPCR142. J Biol Chem *278*, 50765-50770.

Liu, C., Eriste, E., Sutton, S., Chen, J., Roland, B., Kuei, C., Farmer, N., Jornvall, H., Sillard, R., and Lovenberg, T. W. (2003b). Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135. J Biol Chem *278*, 50754-50764.

Liu, J. L., and LeRoith, D. (1999). Insulin-like growth factor I is essential for postnatal growth in response to growth hormone. Endocrinology *140*, 5178-5184.

Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J., and Efstratiadis, A. (1993). Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell *75*, 59-72.

Liu, S. C., Wang, Q., Lienhard, G. E., and Keller, S. R. (1999). Insulin receptor substrate 3 is not essential for growth or glucose homeostasis. J Biol Chem *274*, 18093-18099.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J. L., Bonenfant, D., Oppliger, W., Jenoe, P., and Hall, M. N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell *10*, 457-468.

Louvi, A., Accili, D., and Efstratiadis, A. (1997). Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. Dev Biol *189*, 33-48.

Ludwig, T., Eggenschwiler, J., Fisher, P., D'Ercole, A. J., Davenport, M. L., and Efstratiadis, A. (1996). Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. Dev Biol *177*, 517-535.

Lupu, F., Terwilliger, J. D., Lee, K., Segre, G. V., and Efstratiadis, A. (2001). Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. Dev Biol *229*, 141-162.

Maehama, T., and Dixon, J. E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem *273*, 13375-13378.

Maehama, T., and Dixon, J. E. (1999). PTEN: a tumour suppressor that functions as a phospholipid phosphatase. Trends Cell Biol *9*, 125-128.

Masterson, R., Hewitson, T. D., Kelynack, K., Martic, M., Parry, L., Bathgate, R., Darby, I., and Becker, G. (2004). Relaxin down-regulates renal fibroblast function and promotes matrix remodelling in vitro. Nephrol Dial Transplant *19*, 544-552.

Minami, A., Iseki, M., Kishi, K., Wang, M., Ogura, M., Furukawa, N., Hayashi, S., Yamada, M., Obata, T., Takeshita, Y.*, et al.* (2003). Increased insulin sensitivity and hypoinsulinemia in APS knockout mice. Diabetes *52*, 2657-2665.

Moodie, S. A., Alleman-Sposeto, J., and Gustafson, T. A. (1999). Identification of the APS protein as a novel insulin receptor substrate. J Biol Chem *274*, 11186-11193.

Nandi, A., Kitamura, Y., Kahn, C. R., and Accili, D. (2004). Mouse models of insulin resistance. Physiol Rev *84*, 623-647.

Nef, S., and Parada, L. F. (1999). Cryptorchidism in mice mutant for Insl3. Nat Genet *22*, 295-299.

Nef, S., Verma-Kurvari, S., Merenmies, J., Vassalli, J. D., Efstratiadis, A., Accili, D., and Parada, L. F. (2003). Testis determination requires insulin receptor family function in mice. Nature *426*, 291-295.

Neufeld, T. P. (2004). Genetic analysis of TOR signaling in Drosophila. Curr Top Microbiol Immunol *279*, 139-152.

Neufeld, T. P., de la Cruz, A. F., Johnston, L. A., and Edgar, B. A. (1998). Coordination of growth and cell division in the Drosophila wing. Cell *93*, 1183-1193.

Newsome, T. P., Asling, B., and Dickson, B. J. (2000). Analysis of Drosophila photoreceptor axon guidance in eye-specific mosaics. Development *127*, 851-860.

Ohtsuka, S., Takaki, S., Iseki, M., Miyoshi, K., Nakagata, N., Kataoka, Y., Yoshida, N., Takatsu, K., and Yoshimura, A. (2002). SH2-B is required for both male and female reproduction. Mol Cell Biol *22*, 3066-3077.

Oldham, S., Montagne, J., Radimerski, T., Thomas, G., and Hafen, E. (2000). Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin. Genes Dev *14*, 2689-2694.

Paradis, S., and Ruvkun, G. (1998). Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. Genes Dev *12*, 2488-2498.

Peng, X. D., Xu, P. Z., Chen, M. L., Hahn-Windgassen, A., Skeen, J., Jacobs, J., Sundararajan, D., Chen, W. S., Crawford, S. E., Coleman, K. G., and Hay, N. (2003). Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. Genes Dev *17*, 1352-1365.

Pessin, J. E., and Saltiel, A. R. (2000). Signaling pathways in insulin action: molecular targets of insulin resistance. J Clin Invest *106*, 165-169.

Pierce, S. B., Costa, M., Wisotzkey, R., Devadhar, S., Homburger, S. A., Buchman, A. R., Ferguson, K. C., Heller, J., Platt, D. M., Pasquinelli, A. A.*, et al.* (2001). Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse C. elegans insulin gene family. Genes Dev *15*, 672-686.

Potter, C. J., Huang, H., and Xu, T. (2001). Drosophila tsc1 functions with tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. Cell *105*, 357-368.

Potter, C. J., Pedraza, L. G., and Xu, T. (2002). Akt regulates growth by directly phosphorylating Tsc2. Nat Cell Biol *4*, 658-665.

Puig, O., Marr, M. T., Ruhf, M. L., and Tjian, R. (2003). Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. Genes Dev *17*, 2006-2020.

Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D., and Downward, J. (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature *370*, 527-532.

Rosenzweig, S. A. (2004). What's new in the IGF-binding proteins? Growth Horm IGF Res *14*, 329-336.

Rulifson, E. J., Kim, S. K., and Nusse, R. (2002). Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. Science *296*, 1118-1120.

Saltiel, A. R., and Kahn, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. Nature *414*, 799-806.

Saltiel, A. R., and Pessin, J. E. (2002). Insulin signaling pathways in time and space. Trends Cell Biol *12*, 65-71.

Samuel, C. S., Zhao, C., Bond, C. P., Hewitson, T. D., Amento, E. P., and Summers, R. J. (2004). Relaxin-1-deficient mice develop an age-related progression of renal fibrosis. Kidney Int *65*, 2054-2064.

Sarbassov dos, D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol *14*, 1296-1302.

Scheid, M. P., and Woodgett, J. R. (2001). PKB/AKT: functional insights from genetic models. Nat Rev Mol Cell Biol *2*, 760-768.

Schmelzle, T., and Hall, M. N. (2000). TOR, a central controller of cell growth. Cell *103*, 253-262.

Shaw, R. J., Bardeesy, N., Manning, B. D., Lopez, L., Kosmatka, M., DePinho, R. A., and Cantley, L. C. (2004a). The LKB1 tumor suppressor negatively regulates mTOR signaling. Cancer Cell *6*, 91-99.

Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., and Cantley, L. C. (2004b). The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc Natl Acad Sci U S A *101*, 3329-3335.

Shepherd, P. R., Withers, D. J., and Siddle, K. (1998). Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochem J *333*, 471-490.

Sherwood, O. D., Downing, S. J., Guico-Lamm, M. L., Hwang, J. J., O'Day-Bowman, M. B., and Fields, P. A. (1993). The physiological effects of relaxin during pregnancy: studies in rats and pigs. Oxf Rev Reprod Biol *15*, 143-189.

Simpson, L., and Parsons, R. (2001). PTEN: life as a tumor suppressor. Exp Cell Res *264*, 29-41.

Simpson, P. (1979). Parameters of cell competition in the compartments of the wing disc of Drosophila. Dev Biol *69*, 182-193.

Sjogren, K., Liu, J. L., Blad, K., Skrtic, S., Vidal, O., Wallenius, V., LeRoith, D., Tornell, J., Isaksson, O. G., Jansson, J. O., and Ohlsson, C. (1999). Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. Proc Natl Acad Sci U S A *96*, 7088-7092.

Smith, R. K., Carroll, P. M., Allard, J. D., and Simon, M. A. (2002). MASK, a large ankyrin repeat and KH domain-containing protein involved in Drosophila receptor tyrosine kinase signaling. Development *129*, 71-82.

Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Mak, T. W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell *95*, 29-39.

Staveley, B. E., Ruel, L., Jin, J., Stambolic, V., Mastronardi, F. G., Heitzler, P., Woodgett, J. R., and Manoukian, A. S. (1998). Genetic analysis of protein kinase B (AKT) in Drosophila. Curr Biol *8*, 599-602.

Stephens, L., Eguinoa, A., Corey, S., Jackson, T., and Hawkins, P. T. (1993). Receptor stimulated accumulation of phosphatidylinositol (3,4,5)- trisphosphate by G-protein mediated pathways in human myeloid derived cells. Embo J *12*, 2265-2273.

Sudo, S., Kumagai, J., Nishi, S., Layfield, S., Ferraro, T., Bathgate, R. A., and Hsueh, A. J. (2003). H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2. J Biol Chem *278*, 7855-7862.

Sun, H., Lesche, R., Li, D. M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X., and Wu, H. (1999). PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci U S A *96*, 6199-6204.

Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., and et al. (1994). Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature *372*, 182-186.

Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R., and Yamada, K. M. (1998). Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science *280*, 1614-1617.

Tapon, N., Ito, N., Dickson, B. J., Treisman, J. E., and Hariharan, I. K. (2001). The drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. Cell *105*, 345-355.

Tee, A. R., Manning, B. D., Roux, P. P., Cantley, L. C., and Blenis, J. (2003). Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol *13*, 1259-1268.

Terauchi, Y., and Kadowaki, T. (2002). Insights into molecular pathogenesis of type 2 diabetes from knockout mouse models. Endocr J *49*, 247-263.

Urano, J., Tabancay, A. P., Yang, W., and Tamanoi, F. (2000). The Saccharomyces cerevisiae Rheb G-protein is involved in regulating canavanine resistance and arginine uptake. J Biol Chem *275*, 11198-11206.

Van Slegtenhorst, M., Carr, E., Stoyanova, R., Kruger, W., and Henske, E. P. (2004). Tsc1+ and tsc2+ regulate arginine uptake and metabolism in Schizosaccharomyces pombe. J Biol Chem.

Vanhaesebroeck, B., Leevers, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J., and Waterfield, M. D. (2001). Synthesis and Function of 3-phosphorylated Inositol Lipids. Annu Rev Biochem *70*, 535-602.

Virkamaki, A., Ueki, K., and Kahn, C. R. (1999). Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. J Clin Invest *103*, 931-943.

Watson, R. T., Shigematsu, S., Chiang, S. H., Mora, S., Kanzaki, M., Macara, I. G., Saltiel, A. R., and Pessin, J. E. (2001). Lipid raft microdomain compartmentalization of TC10 is required for insulin signaling and GLUT4 translocation. J Cell Biol *154*, 829-840.

Weigmann, K., Cohen, S. M., and Lehner, C. F. (1997). Cell cycle progression, growth and patterning in imaginal discs despite inhibition of cell division after inactivation of Drosophila Cdc2 kinase. Development *124*, 3555-3563.

White, M. F. (2003). Insulin signaling in health and disease. Science *302*, 1710-1711.

White, M. F., and Yenush, L. (1998). The IRS-signaling system: a network of docking proteins that mediate insulin and cytokine action. Curr Top Microbiol Immunol *228*, 179-208.

Wilkins, A. S. (1995). Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. Bioessays *17*, 71-77.

Williams, M. R., Arthur, J. S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P., and Alessi, D. R. (2000). The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. Curr Biol *10*, 439-448.

Withers, D. J., Gutierrez, J. S., Towery, H., Burks, D. J., Ren, J. M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G. I.*, et al.* (1998). Disruption of IRS-2 causes type 2 diabetes in mice. Nature *391*, 900-904.

Wolkow, C. A., Kimura, K. D., Lee, M. S., and Ruvkun, G. (2000). Regulation of C. elegans life-span by insulinlike signaling in the nervous system. Science *290*, 147-150.

Yakar, S., Liu, J. L., Stannard, B., Butler, A., Accili, D., Sauer, B., and LeRoith, D. (1999). Normal growth and development in the absence of hepatic insulin-like growth factor I. Proc Natl Acad Sci U S A *96*, 7324-7329.

Yamanaka, Y., Wilson, E. M., Rosenfeld, R. G., and Oh, Y. (1997). Inhibition of insulin receptor activation by insulin-like growth factor binding proteins. J Biol Chem *272*, 30729-30734.

Yenush, L., and White, M. F. (1997). The IRS-signalling system during insulin and cytokine action. Bioessays *19*, 491-500.

Zhang, H., Stallock, J. P., Ng, J. C., Reinhard, C., and Neufeld, T. P. (2000). Regulation of cellular growth by the Drosophila target of rapamycin dTOR. Genes Dev *14*, 2712-2724.

Zhang, Y., Gao, X., Saucedo, L. J., Ru, B., Edgar, B. A., and Pan, D. (2003). Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol *5*, 578-581.

Zheng, W. H., Kar, S., Dore, S., and Quirion, R. (2000). Insulin-like growth factor-1 (IGF-1): a neuroprotective trophic factor acting via the Akt kinase pathway. J Neural Transm Suppl *60*, 261-272.

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Publication list

1) Bohni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B. F., Beckingham, K., and Hafen, E. (1999). Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. Cell *97*, 865-875.

2) Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C., and Thomas, G. (1999). Drosophila S6 kinase: a regulator of cell size. Science *285*, 2126-2129.

3) Oldham, S., Bohni, R., Stocker, H., Brogiolo, W., and Hafen, E. (2000). Genetic control of size in Drosophila. Philos Trans R Soc Lond B Biol Sci *355*, 945-952.

4) Stocker, H., and Hafen, E. (2000). Genetic control of cell size. Curr Opin Genet Dev *10*, 529-535.

5) Halfar, K., Rommel, C., Stocker, H., and Hafen, E. (2001). Ras controls growth, survival and differentiation in the Drosophila eye by different thresholds of MAP kinase activity. Development *128*, 1687-1696.

6) Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., and Hafen, E. (2001). An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. Curr Biol *11*, 213-221.

7) Clancy, D. J., Gems, D., Harshman, L. G., Oldham, S., Stocker, H., Hafen, E., Leevers, S. J., and Partridge, L. (2001). Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. Science *292*, 104-106.

8) Rintelen, F., Stocker, H., Thomas, G., and Hafen, E. (2001). PDK1 regulates growth through Akt and S6K in Drosophila. Proc Natl Acad Sci U S A *98*, 15020-15025.

9) Stocker, H., Andjelkovic, M., Oldham, S., Laffargue, M., Wymann, M. P., Hemmings, B. A., and Hafen, E. (2002). Living with lethal PIP3 levels: viability of flies lacking PTEN restored by a PH domain mutation in Akt/PKB. Science *295*, 2088-2091.

10) Radimerski, T., Montagne, J., Rintelen, F., Stocker, H., van der Kaay, J., Downes, C. P., Hafen, E., and Thomas, G. (2002). dS6K-regulated cell growth is dPKB/dPI(3)K-independent, but requires dPDK1. Nat Cell Biol *4*, 251-255.

11) Nairz, K., Stocker, H., Schindelholz, B., and Hafen, E. (2002). High-resolution SNP mapping by denaturing HPLC. Proc Natl Acad Sci U S A *99*, 10575-10580.

12) Oldham, S., Stocker, H., Laffargue, M., Wittwer, F., Wymann, M., and Hafen, E. (2002). The Drosophila insulin/IGF receptor controls growth and size by modulating PtdInsP(3) levels. Development *129*, 4103-4109.

13) Gopfert, M. C., Stocker, H., and Robert, D. (2002). atonal is required for exoskeletal joint formation in the Drosophila auditory system. Dev Dyn *225*, 106-109.

14) Stocker, H., Radimerski, T., Schindelholz, B., Wittwer, F., Belawat, P., Daram, P., Breuer, S., Thomas, G., and Hafen, E. (2003). Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. Nat Cell Biol *5*, 559-565.

15) Garami, A., Zwartkruis, F. J., Nobukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S. C., Hafen, E., Bos, J. L., and Thomas, G. (2003). Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell *11*, 1457-1466.

16) Junger, M. A., Rintelen, F., Stocker, H., Wasserman, J. D., Vegh, M., Radimerski, T., Greenberg, M. E., and Hafen, E. (2003). The Drosophila Forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J Biol *2*, 20.

17) Hafen, E., and Stocker, H. (2003). How Are the Sizes of Cells, Organs, and Bodies Controlled? PLoS Biol *1*, E86.

18) Ryder, E., Blows, F., Ashburner, M., Bautista-Llacer, R., Coulson, D., Drummond, J., Webster, J., Gubb, D., Gunton, N., Johnson, G.*, et al.* (2004). The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in Drosophila melanogaster. Genetics *167*, 797-813.

19) Reiling, J. H., Doepfner, K. T., Hafen, E., and Stocker, H. Diet-dependent effects of the *Drosophila* Mnk1/Mnk2 homolog Lk6 on growth via eIF4E. Current Biology, in press.